Ex-vivo Lung Perfusion: a platform for assessment and reconditioning of human donor lungs.

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Abstract

Ex-vivo Lung Perfusion: a platform for assessment and reconditioning of human donor lungs.

Background: Although lung transplantation is regarded as an effective therapy for patients with end-stage lung disease, the demand for donor organs exceeds the overall donor organ supply. *Ex-vivo lung perfusion* (EVLP) has emerged as a technique to objectively assess and recondition donor lungs that have been deemed unsuitable for transplantation. The cellular and molecular events occurring during EVLP have not been well characterised, and how these processes differ between lungs that are deemed useable compared with those that are discarded is unknown. The aims of this project were three-fold. Firstly, to identify potential predictive biomarkers during EVLP that could distinguish donor lungs that can be reconditioned and predict clinical outcomes. Secondly, to investigate the differences in the inflammatory profiles of donor lungs following donation after brain death (DBD) versus donation after circulatory death (DCD). Finally, to assess EVLP as a therapeutic platform by examining the physiological and immunological changes in donor lungs undergoing EVLP supplemented with the known vasodilatory agent and phosphodiesterase-5 inhibitor, sildenafil.

Methods: In retrospective studies of the DEVELOP-UK cohort and prospective studies using a research EVLP model, markers of inflammation, tissue injury and angiogenesis in perfusate, bronchoalveolar lavage (BAL), and lung tissue were examined. Laboratory techniques including ELISA, MSD and immunohistochemistry were used.

Results: RNA-sequencing demonstrated an unbiased profile of the transcriptome of human donor lungs prior to, and following EVLP, comparing changes in gene expression between lungs deemed suitable for transplantation, and those discarded on the basis of standard physiological parameters. During EVLP, we observed an increase in a number of immune pathway genes.

In the DEVELOP-UK sample cohort significant differences were detected in the inflammatory profiles prior to commencing EVLP between DBD and DCD donor lungs. DBD donor lungs were reported to have significantly higher concentrations of lung tissue C-reactive protein (CRP), IL-12p70, IL-4 and IL-6 compared with DCD

donor lungs. Conversely, the levels of acute phase cytokines, IL-1β and IL-1α, were significantly higher in the perfusate from DCD lungs during EVLP.

EVLP was shown to be an effective and reproducible therapeutic platform which could allow for the testing of agents with the potential for improving the function of lungs turned down for transplantation. Sildenafil was hypothesised to improve pulmonary physiology during EVLP and lead to improved oxygenation and pulmonary vascular homeostasis, however this did not occur. Sildenafil did not have any significant effects on the physiology and immune profile of human donor lungs during EVLP.

Discussion: In order for EVLP is to be effectively utilised in clinical practice as a platform for the assessment, reconditioning and potential treatment of donor lungs, an increased understanding of the cellular and molecular events occurring is required. In this study, we aimed to address this knowledge gap by performing an unbiased analysis of the lung transcriptome prior to and following EVLP. RNA-sequencing can be used to study the pro-inflammatory changes within the lung transcriptome during EVLP, with subsequent correlation to potential protein biomarkers in perfusate. We reported significant differences in the inflammatory profiles of DBD versus DCD lungs. Finally, administration of sildenafil into the vascular compartment of the EVLP circuit failed to produce the hypothesised beneficial effects in human donor lung physiology and function.

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List of Abbreviations

ABG - Arterial blood gas

ABO - ABO classification system for human blood

ALI - Acute lung injury

ANOVA - Analysis of variance

APCs - Antigen presenting cells

AR - Acute rejection

ARDS - Acute Respiratory Distress

Syndrome ATP - Adenosine 5′-triphosphate

BAL - Bronchoalveolar lavage

BOS - Bronchiolitis Obliterans Syndrome

BSA - Bovine serum albumin

BTRU - Blood and Transplant Research Unit

Cdyn - Dynamic pulmonary compliance

CF - Cystic Fibrosis

CIT - Cold ischaemic time

cGMP - Cyclic guanosine monophosphate

CMV - Cytomegalovirus

CO - Cardiac Output

CO2 - Carbon dioxide

COPD - Chronic Obstructive Pulmonary Disease

CPB - Cardiopulmonary bypass

CPR - Cardio-Pulmonary Resuscitation

CXR - Chest radiograph

DAB - 3,3′-Diaminobenzidine

DAMPs - Damage associated molecular patterns

DBD - Donation after Brain Death

DCD - Donation after Circulatory Death

DEVELOP- UK

DLTx - Double-Lung Transplantation

E. coli - Escherichia coli

ECD - Extended criteria donor

ECMO - Extra-Corporeal Membrane Oxygenation

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-linked immunosorbent assay

eNOS - Endothelial nitric oxide synthase

ET-1 - Endothelin-1

EVLP - Ex Vivo Lung Perfusion

EVNP - Ex vivo normothermic preservation

FiO2 - Fraction of inspired oxygen

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GSEA - Gene Set Enrichment Analysis

GTN - Glyceryl trinitrate

H. influenzae - Haemophilus influenzae

HA1AGP - Human Alpha – 1 acid Glycoprotein

HAECs - Human Amniotic Endothelial Cells

HMGB1 - High mobility group box protein -1

HCT - Haematocrit

HCU - Heater cooler unit

HRP- Horseradish peroxidase

HSP-70 - Heat Shock Protein 70

HTA - Human Tissue Authority

IBW- Ideal body weight

ICAM-1- Intercellular adhesion molecule-1

ICP - Intracranial pressure

ICU - Intensive Care Unit

IFN-γ - Interferon-gamma

IL - Interleukin

IL- 2 - Interleukin 2

IL-10- Interleukin 10

IL-1β - Interleukin 1-Beta

IL-6 - Interleukin 6

IPF - Idiopathic pulmonary fibrosis

IRI - Ischaemia-reperfusion injury

ISHLT - International Society of Heart and Lung Transplantation

LA - Left Atrium

LDH - Lactate dehydrogenase

LLOD - Lower limit of detection

LPD - Low potassium dextran

LTx - Lung Transplantation

MAP - Mean arterial pressure

MHC - Major histocompatibility complex

MIG - Monokine induced by gamma interferon

MIP 3-Beta - Macrophage inflammatory protein 3 beta

mmHG - Millimetres of mercury

MPAP - Mean pulmonary artery pressure

mRNA - Messenger ribonucleic acid

MSCs - Mesenchymal Stem Cells

MSD - Meso scale discovery

mtDNA - Mitochondrial DNA

N2 - Nitrogen

NE - Neutrophil elastase

NF-κB - Nuclear factor Kb

NHS - National Health Service

NHS-BT - NHS Blood and Transplant

NO - Nitric oxide

NORS - National Organ Retrieval Service

O2 - Oxygen

OCS - The Organ Care System

P. aeruginosa - Pseudomonas aeruginosa

P/F ratio - PaO2/FiO2 ratio

PA - Pulmonary artery

PAH - Pulmonary Arterial Hypertension

PaO2 - Partial pressure arterial oxygen

PAP - Pulmonary Artery Pressure

PawP - Peak airway pressure

PBS - Phosphate-buffered saline

PCO2 - Partial pressure of carbon dioxide

PDE-5 - Phosphodiesterase 5

PEEP -Positive end-expiratory pressure

PF - Pulmonary Fibrosis

PGD - Primary Graft Dysfunction

PGE-1 - Prostaglandin-E1

PIGF - Placental growth factor

PKC - Protein Kinase C

PKG - Protein Kinase G

PMN - Polymorphonuclear neutrophil

PTX – 3 - Pentraxin 3

PVR - Pulmonary Vascular Resistance

RBC - Red Blood Cell

rcPOC - Recipient centre point of contact

RINTAG - Research, Innovation and Novel Technologies Advisory Group

RNA – Ribonucleic acid

ROS - Reactive oxygen species

RR - Respiratory rate

SCS - Static cold storage

sGC - Soluble guanylate cyclase

SD – Standard deviation

SEM - Standard error of the mean

sFlt-1 - Soluble fms-like-tyrosine kinase-1

SIRS - Systemic inflammatory response syndrome

SLTx - Single lung transplantation

SNOD - Specialist nurses for organ donation

THAM - Tromethamine

TLC – Total lung capacity

TNFα - Tumour necrosis factor alpha

TV - Tidal volume

U - Units

VCAM-1 - Vascular cell adhesion molecule-1

VEGF - Vascular endothelial growth factor

W/D - Wet to Dry Ratio

WLST - Withdrawal of life sustaining therapy

List of Presentations

1. Oral presentation: *Biomarker profiling during Ex Vivo Lung Perfusion.* 6th Annual Lung Transplant Biospecimen Workshop, Nice, France, 10th April 2018.

2. Platform oral presentation: *A comparison of inflammatory profiles in donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD).*38th Annual Meeting of the Institute of Heart and Lung Transplantation (ISHLT), Nice France, 13th April 2018.

3. Oral presentation: *Resuscitating & reconditioning donor lungs ex vivo.* BTRU Public Engagement Event, Institute of Transplantation, Freeman Hospital, Newcastle 7th February 2018

4. Teaching seminar: *Ex-vivo Lung Perfusion: A novel platform for assessment and treatment of human lungs deemed unfit for transplantation.* NHS Blood Donor Centre, Holland Drive, Newcastle, 5th March 2018. Specialist Nurses in Organ Donation (SNOD) training (10 attendees)

5. Oral presentation: *Predictive biomarkers in ex vivo lung perfusion.* The 3rd Annual Perfusion Symposium, Institute of Transplantation, Freeman Hospital, Newcastle, 30th June 2018

5. Oral presentation: *Ex-vivo lung perfusion.* The UK Regenerative Medicine Platform/NHS Blood and Transplant (UKRMP/NHS-BT) Joint workshop, 30th May 2017

6. Oral presentation: *Identifying potential predictive biomarkers of successful Ex-vivo Lung Perfusion*. BTRU Trainees Meeting, University of Cambridge, 22nd March 2017. Won First Prize for oral presentation.

List of Publications

1. Published manuscript: Morrison MI, Pither TL, Fisher AJ. Pathophysiology and classification of primary graft dysfunction after lung transplantation. *Journal of Thoracic Disease*. 2017;9(10):4084-4097. doi:10.21037/jtd.2017.09.09.

2. Manuscript in final draft: Transcriptional analysis of the molecular pathways associated with successful pre-transplant ex-vivo lung perfusion reveals potential novel biomarkers John R. Ferdinand*, Morvern I. Morrison*, Anders Andreasson, Alisha Chhatwal, William E. Scott, Lee A. Borthwick, Menna Clatworthy, Andrew J. Fisher

3. Manuscript in final draft: A comparison of Inflammatory profiles in donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD). Morvern M*, Anders Andreasson*, Lee Borthwick, WE Scott III, John Dark, Andrew Fisher.

4. Published abstract: *A comparison of inflammatory profiles in donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD). Oral presentation at* 38th Annual Meeting of the Institute of Heart and Lung Transplantation (ISHLT), Nice France, 13th April 2018.

5. Published abstract: *Identifying potential predictive biomarkers of successful Ex- vivo lung perfusion (EVLP).* Poster presentation at 38th Annual Meeting of the Institute of Heart and Lung Transplantation (ISHLT), Nice France, 13th April 2018.

CHAPTER 1

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Introduction

Chapter 1. Introduction

Clinical Lung Transplantation

The History of lung transplantation

Organ transplantation was one of the most significant medical achievements of the twentieth century and spans the fields of science, medicine, surgery, ethics, and law. Dr James Hardy reported the first successful human lung transplant, in Mississippi on the 11th June 1963; however, the patient died from kidney failure only eighteen days postoperatively[1]. The immunosuppressive regimen consisted of azathioprine, prednisolone, and cobalt radiation to the mediastinum and thymus. Notably, cyclosporine and tacrolimus, which are the cornerstones of immunosuppression in modern transplantation, had not yet been discovered. The first real survivor during this early era of lung transplantation was a patient of Fritz Derom, in Belgium[2].This patient survived ten and a half months but, unfortunately, was the only patient to benefit from lung transplantation prior to 1980. Inadequate immunosuppression and problems with the bronchial anastomosis were the primary reasons for poor outcomes in the early cases of lung transplantation. In 1976, Jean Francoise Borel discovered cyclosporin, which changed the history of transplantation forever[3]. The significant improvements in patient survival following liver and kidney transplantation[4] due to cyclosporin led to a renewed interest in cardiothoracic transplantation. Bruce Reitz performed the first successful heart and lung transplantation in 1981[5] and research done by Joel Cooper's group in Toronto showed that corticosteroid use was a significant factor in causing the dehiscence of the bronchial anastomosis. In 1983 cyclosporin became a clinically approved therapy to prevent graft rejection in transplantation and subsequently corticosteroid use could be reduced, leading to improved bronchial anastomosis. In 1986, Cooper reported the first successful single-lung transplantations for two patients with pulmonary fibrosis[6, 7]. His team went on to perform successful double-lung transplants, first with an *en-bloc* technique, then with a bilateral-sequential transplantation technique which improved airway healing and had the additional benefit of avoiding cardiopulmonary bypass; if desired. This technique remains mostly in use to this day.

The lung transplant recipient

Lung transplantation has evolved into a safe and effective treatment of severe progressive end-stage lung disease, where all medical and surgical therapies have been exhausted. The lung transplant waiting list contains potential recipients with a diverse range of lung pathologies[8]. In the United Kingdom (UK), between 1 April 2016 and 31 March 2017, 56% of patients were male and the median age was 52 years. The indications for lung transplantation can be broadly separated into the following main categories of end-stage lung diseases: obstructive lung disease, septic lung disease, fibrotic lung disease, and vascular lung disease. Of these categories, chronic obstructive pulmonary disease (COPD), cystic fibrosis(CF), fibrosing lung disease, and primary pulmonary arterial hypertension (IPAH) make up the most common indication in each category, respectively **(Table 1.1)**.

Indication	Percentage of all recipient diagnoses (%)
Chronic obstructive pulmonary disease (COPD)/ emphysema	26
Fibrosing lung disease	26
Cystic fibrosis (CF) and bronchiectasis	28
Idiopathic pulmonary arterial hypertension (IPAH)	3
Other	17

Table 1.1 Indications for lung transplantation

Table shows the common indications for lung transplantation and the corresponding percentages. Adapted from NHS Blood and Transplant Cardiothoracic Report 2016/2017[9].

The lung transplantation procedure carries important risks to the often frail transplant recipient with an approximate 10% 90-day mortality risk in the UK[10]. The fatal complications that can occur post lung transplantation vary depending on the time since the transplant procedure. Whereas 30-day mortality is generally related to surgical issues, donor lung preservation, and primary graft dysfunction (PGD): infectious causes, malignancy, and bronchiolitis obliterans syndrome (BOS), a type of chronic rejection, predominate after the early post-transplant period. The appropriate selection of lung transplant recipients is an important determinant of outcomes and the selection process for choosing patients to go onto the waiting list is, therefore, strict[11].

To address this The Pulmonary Council of the International Society for Heart and Lung Transplantation (ISHLT) published the Third Edition of the Consensus Report for the Selection of Lung Transplant Candidates[12]. The respiratory physician has a difficult decision to make in listing a patient on the active lung transplant waiting list. It is a decision that must take into account several factors including timing, treatments given to date, co-morbidities, frailty and the risk of surgery. By listing a patient for a lung transplant, both the patient and clinician recognise the limited life expectancy and poor quality of life associated without receiving a transplant. Patients, who are wait-listed for lung transplantation are at significant risk of morbidity and mortality. On the lung transplant waiting list, there is a 12% 1-year mortality, rising to 17 % after 3-years. On 31st March 2017, there were 360 patients on the active UK lung transplant waiting list and 18 on the heart-lung list, a 32% increase since 2008[13]. Despite this, the number of lung or heart-lung transplants carried out between 2016 and 2017 however, fell by 5% to 178. The national median waiting time on the lung transplant waiting list is 255 days, and unfortunately, patients are often removed from the waiting list due to being too unwell for transplantation, **Figure 1.1**.

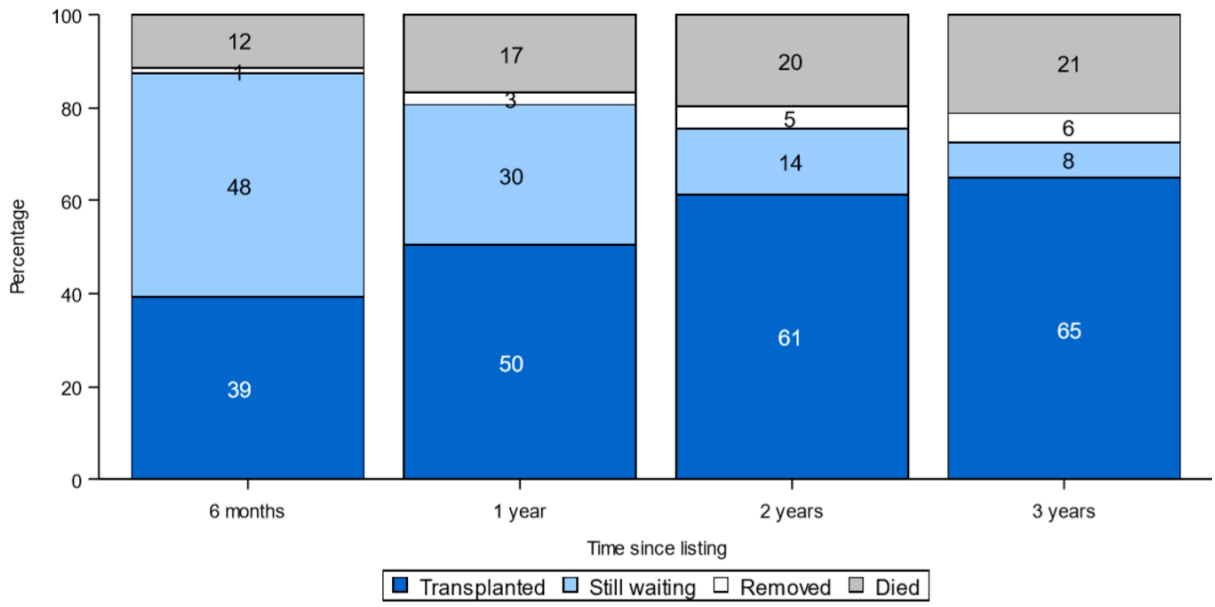

Figure 1.1 The UK adult lung transplant waiting list

Adapted from the NHS-BT Annual Report for Cardiothoracic Activity 2016/2017. This bar chart shows the percentage of patients transplanted (dark blue) or still waiting (light blue) six months, one year, two years and three years after joining the non-urgent lung list, respectively. It also shows the percentage of patients removed from the transplant list (white) and those that died (grey) whilst on the transplant list. Within six months of listing, 39% of lung patients had been transplanted and 12% had died. Three years after listing 65% have been transplanted and 21% had died. The patients removed from these lists may also subsequently have died[13].

Utilisation of lung from organ donors

The number of lung transplantations that can be performed is directly related to the number of donor lungs available for transplantation, and a shortage of donor lungs restricts this. There were 1,574 actual deceased organ donors in 2017-2018, but not all organs from these donors were offered for transplantation. There were 955 donors after brain death (DBD) and 619 donors after circulatory death (DCD). Compared with other solid organs, lungs have a very poor conversion rate from potential donors, to lungs being offered, to lungs transplanted. Although a shortage of multi-organ donors contributes, a central concern in cardiothoracic transplantation is the high susceptibility of donor lungs to injury and dysfunction during the time of the donors' demise and organ procurement. Many potential donor lungs are deemed unsuitable and excluded from use by the clinical selection criteria. During the 2017/2018 period,

lungs were offered for transplant in only 56.5% of DBD donors. In 20 % of donors, their lungs were deemed suitable for use in clinical transplantation, and subsequently retrieved; and only 16% transplanted, **Figure 1.2**[13]. In DCD donors, which make up ~40% of all donors in the UK, only 6% result in transplantation. Despite consent being given from family members and next of kin, a large number of donor lungs are not used for transplant.

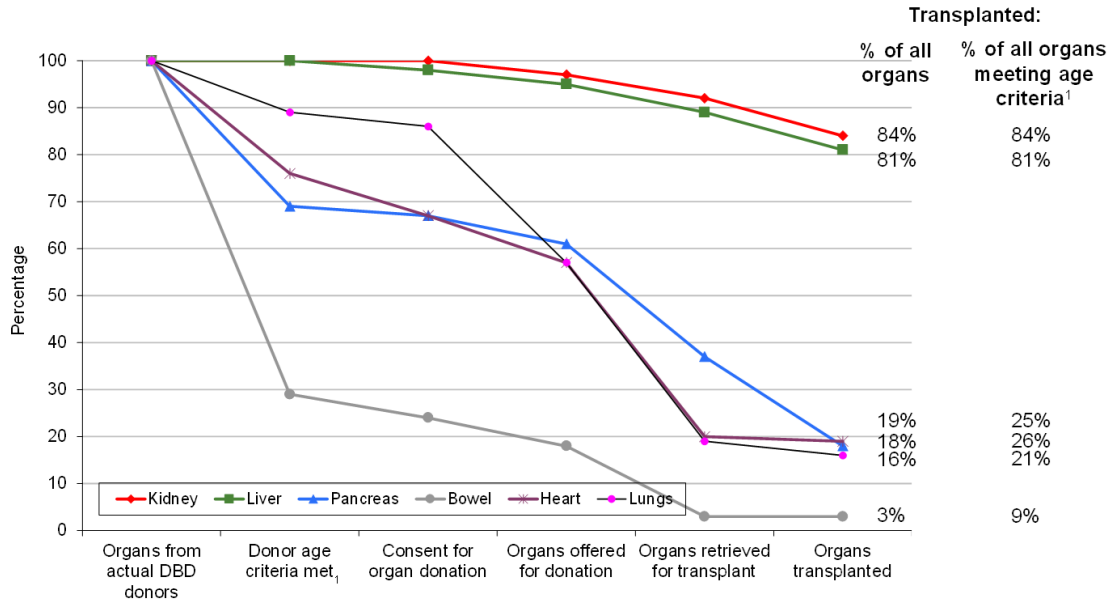

Figure 1.2 Donation and transplantation rates of organs from DBD organ donors in the UK

Adapted from the NHS-BT Report "The National Organ Retrieval Service and Usage of Organs" 2017/2018[14]. Line graphs of the pathway for all donor organs through to transplantation. The charts start at 100% for each organ, representing all organs from the 955 DBD and 619 DCD donors. The proportion of these organs where any national donor age criteria are met is then shown, followed by the proportion with consent, the proportion offered, the proportion retrieved and finally the proportion transplanted. Comparisons can be seen between kidneys (red), livers (green), pancreas (blue), bowel (grey), heart (purple) and lung (pink). Transplantation rates for kidneys and livers are generally high, while for lungs, even after allowing for the agreed age criteria, the rates are generally low.

National publicity campaigns have yet to show any striking increase in the rates of organ donation and other strategies are needed to increase the number of lung transplantations being carried out. There have been various implementations and changes in the transplant culture towards expanding the lung donor pool; which include extending the donor criteria and using DCD donors[15, 16]. The number of

transplants from DBD donors has generally increased since 2007 from 111 to 146 in 2015/2016, while the number of transplants from DCD donors has substantially increased from only 3 in 2006 to 33 in 2015/2016[9]. Despite increasing numbers of DCD donors since 2008, this resource has failed to make a significant impact on the overall numbers of lung donors; highlighting that further strategies to increase the lung donor pool are needed.

Selection criteria for Donation – The "Ideal Donor"

Currently, less than 20% of the DBD lungs that are offered in the UK are actually transplanted, while 80% of the remaining donor lungs are not accepted by the transplant programs primarily due to being deemed unsuitable before retrieval[9]. It is therefore vital to assess the way in which potential donor lungs are being evaluated for their function and suitability for transplantation[17]. In the UK, lungs and other organs intended for transplantation, are currently evaluated subjectively 'by a trained eye' alongside selection criteria, which have been shown to be poor discriminators of injury and infection. When donor lungs are offered to an institution, blood samples are obtained to check the blood group and to minimize the risk of donor-transmitted diseases. The size of the donor is then considered and a potential recipient is chosen based upon size and blood group. A chest x-ray (CXR) is taken to exclude gross parenchymal or pleural abnormalities, and bronchoscopy is performed to exclude gross infection or anatomical abnormalities. Finally, the gas exchange capacity of the donor lungs is assessed with an oxygen challenge. At retrieval, the surgeon performs a gross physical evaluation by macroscopic observation and palpation to assess lung compliance and oedema[18]. Palpation is also used to exclude intrinsic lung disease, areas of contusion, pneumonic infiltrates, or nodules. Observation of the ventilated lungs during deflation is used to assess pulmonary compliance. This crude method leads to the unavoidable exclusion of potentially useable lungs in the name of safety-related conservatism. The majority of organ donors arise from patients who have suffered severe brain injury[19]. Multi-organ donors may become brain dead as a result of any number of causes; head trauma, cerebrovascular accidents, including spontaneous intracerebral bleeding and thrombosis, anoxic, metabolic or toxic brain injury. Lungs from brain-dead donors are fragile and more sensitive to traumatic situations compared with other organs. Lungs may get injured in the hours leading to and after brain death resulting from direct trauma, resuscitation manoeuvres, neurogenic oedema, aspiration of blood or gastric content, ventilator-associated trauma and pneumonia, making them unsuitable for

transplantation. Utilising donor lungs which do not completely satisfy the criteria is an approach that could potentially expand the donor lung pool.

The current International Society for Heart and Lung Transplantation (ISHLT) criteria outlining an ideal donor aims to establish safe, but conservative, clinical lung transplantation[20]. The ideal lung donor is young, previously healthy, non-smoking, and without impairment of lung function in the period preceding donation, **Table 1.2**.

ISHLT Criteria for Lung Acceptance
• Age <55 years
• ABO compatibility
• Clear chest radiograph
• PaO2>300 on FiO2 = 1.0, PEEP 5 cm H20
• Tobacco history <20 pack-years
• Absence of chest trauma
• No evidence of aspiration/sepsis
• No prior cardiopulmonary surgery
• Sputum gram stain – absence of organisms
• Absence of purulent secretions at bronchoscopy

Table 1.2 ISHLT Criteria for Lung Acceptance

The "Ideal" Lung Donor according to ISHLT guidelines. PaO2 denotes partial pressure of oxygen, FiO2 fraction of inspired oxygen, PEEP peak end-expiratory pressure. Adapted from Orens et al.[11]

The ISHLT recommended, that one must consider the individual donor to have several factors including age, graft ischaemic time, blood type ABO compatibility, sex, organ size matching, cause of death, duration of mechanical ventilation, radiographic findings, presence of microbial organisms, arterial blood gases, smoking exposure, and donor diagnoses of malignancy that may affect post-transplant lung function. These factors should not be viewed in isolation but rather as interacting variables on a spectrum of donor lung acceptability, from the ideal donor over the extended donor to the unusable donor. What had previously been regarded as the

“ideal lung donor” by the ISHLT, no longer reflects what is considered acceptable in most centres. Several studies in the mid to late 1990s retrospectively assessed the outcomes and early graft function in recipients of non-ideal or “marginal” donor lungs and reported no significant difference in early graft function between the two groups[21-23], suggesting that a large number of potential donor lungs which are currently excluded; may have been successfully used in lung transplantation. Fisher *et al.* in 2004 studied 39 consecutive potential donor lungs, 14 of which were accepted and 25 excluded by clinical selection criteria[24]. All were evaluated prospectively by clinical assessment, bronchoscopy, and bronchoalveolar lavage (BAL) to evaluate the discrimination of pulmonary infection and injury objectively. Accepted donors were significantly younger than those excluded (mean (SD) age 36.7 (15.3) years v 49.5 (13.2) years; p=0.009) and were more likely to have suffered traumatic brain death (50% v 20%; p=0.07, Fisher’s exact test). They found that positive formal BAL cultures were more frequent in accepted donors (75%) than in those excluded (43%). There was no significant difference in the percentage and concentration of neutrophils, or the concentration of inflammatory cytokines, Tumour Necrosis Factor-α (TNF-α) and Interleukin-8 (IL-8) in BAL fluid between accepted and excluded donors. This study highlighted that the current selection criteria are poor discriminators of pulmonary injury and infection and ultimately lead to the exclusion of potentially usable donor lungs.

Donors that do not meet the “ideal” criteria, also called “marginal donor lungs” or “non-ideal donor lungs,” are not systematically used for transplantation. However, these “marginal donors” are not systematically discarded either. Van Raemdonck *et al.* published in 2009, that “marginal” donor lungs are used in 33% to 77% of cases, as reported in published series from the U.S., Canada and Europe[25]. This study demonstrates the considerable variability between transplant centres internationally and the need for more consensus opinion.

Meers *et al.* reported on all consecutive deceased organ donors within the Leuven hospital network over a 2-year period with the reasons for lung refusals and number of lungs transplanted being analysed[26]. Hospital outcome including early recipient survival was compared between standard and extended criteria donors. Out of 283 referrals, 164 (58%) qualified as donors of any organ. The majority (65.9%) of these effective donors were declined for lung donation because of CXR abnormalities

(20%), age >70 years (13%), poor oxygenation (10%), or aspiration (9%). Out of 56 (34.1%) accepted lung donors, 50 transplants were performed, 23 from standard criteria donors versus 27 from extended criteria donors. There were no significant differences in hospital outcome or early survival between lung recipients from both donor groups. This group concluded from their study that relaxing the standard lung donor criteria may significantly increase the reported 15% organ yield, but the importance of monitoring post-transplant recipient outcome should not be underestimated.

The burden of lungs being deemed “marginal” has been emphasised by several groups internationally. Cypel *et al.* in Toronto considers that more than 80% of donor lungs are potentially injured and therefore not considered suitable for transplantation[27]. Several reports have suggested equivalent outcomes when standard and marginal donors are used. Ware *et al.* estimated that over 41% of the lungs rejected for transplantation could have been used[28].

The ultimate judgment as to whether a donor lung is used for transplantation is made on the basis of donor and recipient factors in each individual case. There is a clear need for the extension of the traditional donor criteria to help ease the profound shortage of donor lungs. However, follow-up studies are necessary to validate the safety and efficacy of broader acceptability criteria. If we can increase the utilisation of currently available potential lung donors, this will have a significant impact on the imbalance between transplants performed and the rate of deaths while on the waiting list.

Mechanisms of Donor Lung Injury

Lung function is often impaired in organ donors, even in patients with previously healthy lungs. Several factors may contribute to this phenomenon, such as mechanical ventilation, aspiration, trauma, infections, and pulmonary oedema[29]. In DBD donors, the pathophysiology of lung injury following brain death is multifactorial**, Figure 1.3.**

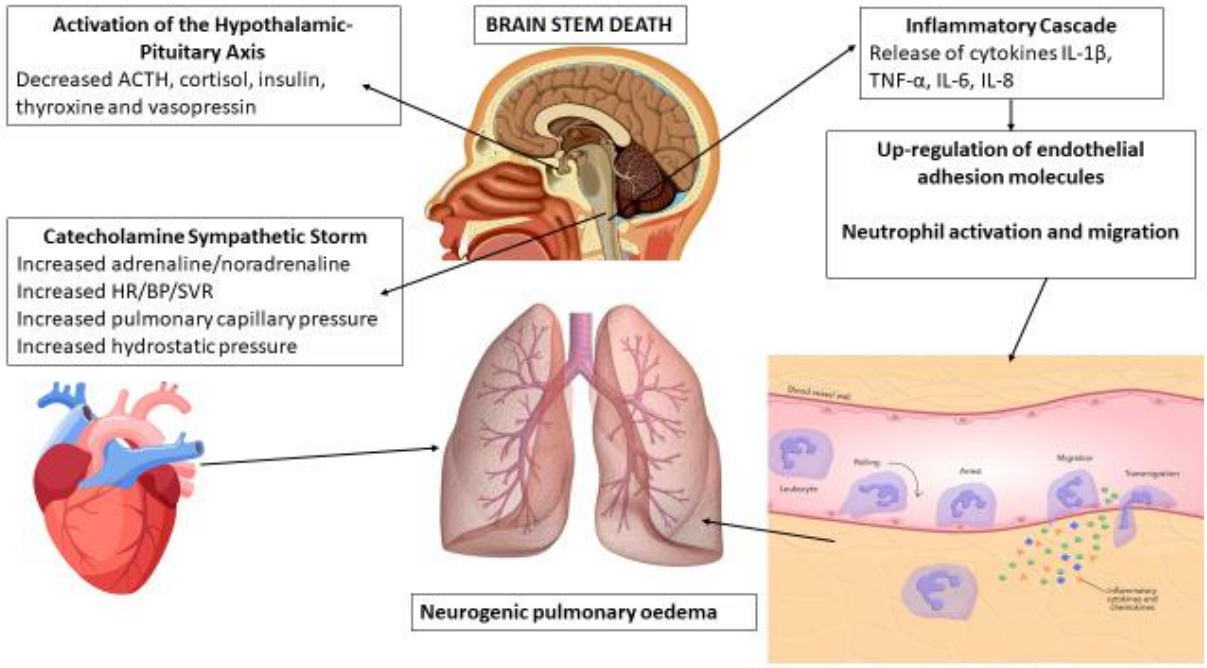

Figure 1.3The pathophysiology of lung Injury in brainstem death

Schematic diagram demonstrating the multifactorial pathophysiology of lung injury following brainstem death. ACTH: adrenocorticotropic hormone; IL: interleukin; TNF: tumour necrosis factor; HR: heart rate; BP: blood pressure; SVR: systemic vascular resistance.

Brain death results from damage to the brain stem with complete, irreversible loss of its function. Donors become brain dead following a rise in intracranial pressure (ICP) owing most commonly to either massive intracranial haemorrhage or head trauma[30]. This increased pressure in the skull leads to cerebral venous engorgement and brain swelling, which further increases ICP. As the pressure increases, the brain stem is pushed through the foramen magnum, leading to arterial compression and brain infarction, commonly known as coning[31]. Coning results in even more brain swelling and increases ICP to the point of ceasing intracranial circulation and brain death. The autonomic response to elevated ICP triggers pulmonary oedema. The underlying mechanism of neurogenic pulmonary oedema is not completely understood but most likely depends on: a) neurocardiac effects, whereby massive release of catecholamine induces direct myocyte injury and fluid congestion in the lungs; b) neuro-haemodynamic effects as the systemic and pulmonary pressures increase after brain injury; and c) increased permeability of the capillary-alveolar membranes, caused by the increase in blood volume in the pulmonary vessels[32].

There is an initial phase of brain stem stimulation resulting in sympathetic discharge producing a ‘catecholamine storm’ and hypertensive crisis. This is followed by loss of vasomotor tone and neurogenic hypotension. Brain death also causes significant endocrine changes involving the hypothalamic-pituitary axis[33]. Adrenocorticotrophic hormone, cortisol, triiodothyronine, thyroxine, insulin, and vasopressin plasma levels decrease[34]. The catecholamine storm, endocrine and hemodynamic disturbances, and consequential organ hypoperfusion result in metabolic derangement[35, 36]. There is a shift from aerobic to anaerobic metabolism, with depletion of the energy stores and lactic acidosis. The release of destructive enzymes such as lipases, proteases, endonucleases and the generation of ROS can start at this stage; leading to further insult. At the same time, the immune system is activated with the development of a profound systemic inflammatory response. Upregulation of pro-inflammatory cytokines is observed in most organs[37-39]. Pulmonary endothelial activation is key in the infiltration and activation of neutrophils into the lungs, resulting in local tissue injury and acute lung injury (ALI). It is partly caused by the pro-inflammatory signals as a result of brain stem death[40]. Cytokines play a crucial role in this inflammatory response, brain death in rats results in the up-regulation of interleukin- 1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), TNF-α, and interferon γ (IFN-γ) in multiple organs including the lungs[37]. Korovesi *et al.* observed both pulmonary and systemic inflammation in patients who required mechanical ventilation for severe brain injury. Brain dead patients exhaled elevated levels of interleukin-1β (IL-1β), IL-6, and TNF-α compared to healthy controls, with cytokine expressions strongly correlated to plasma IL-8 concentrations. They also had characteristic changes in lung mechanics suggesting subclinical pulmonary inflammation before they became possible organ donors[41]. De Perrot *et al*. showed that IL-8 levels in donor lung tissue before and after transplantation increased with time following reperfusion and that patients who developed severe primary graft dysfunction had significantly higher IL-8 levels during ischemia and after reperfusion[42].

Similarly, Fisher *et al.* studied the levels of IL-8 in bronchoalveolar lavage(BAL) fluid from 26 donor lungs used for transplantation and showed that a high concentration of IL-8 in donor BAL was correlated with severe graft dysfunction and early postoperative deaths[43]. Kaneda *et al*. further studied the role of pro-inflammatory cytokines by using real-time reverse transcriptase polymerase chain reaction (RT-PCR) to study the levels of IL-6, IL-1β, IL-8, IL-10, IFN -γ, and TNF-α in the donor

lung at the end of cold ischemia and found that the IL-6/IL-10 ratio was predictive of recipient 30-day mortality[44]. ALI itself results in microvascular endothelium response to local production of IL-1β or TNF-α, leading to expression of endothelial cell-derived E-selectins and ICAM-1 in a positive feedback manner[45]. The lung is acutely sensitive to the above changes[19], enabling neutrophils to bind to endothelial cells and migrate into the interstitium and alveolar spaces of the lung, where they can cause further injury through the release of ROS and proteolytic enzymes[45].

In order to maximize the utilisation of lugs from multi-organ donors and improve recipient outcomes, a strategy of aggressive donor management must be implemented[46]. The aim should be a reduction of donor lung injury and prevention of its amplification. Donor management measures such as protective ventilation adopting optimal pressure control ventilation, physiotherapy, bronchial toilet, invasive monitoring, hemodynamic support, judicious fluid management, maintenance of adequate diuresis, and antibiotics have all been shown to achieve excellent recipient results with lungs previously regarded as unacceptable[47-51].

Primary graft dysfunction

Following ischaemia in the donor organ and reperfusion in the recipient, inflammatory and immunological injury-repair responses seem to be the key pathological mechanisms. Primary graft dysfunction (PGD) is the syndrome that encompasses a spectrum of mild to severe lung injury that occurs within the first 72 hours after lung transplantation. PGD is a major cause of early morbidity and mortality in lung transplantation and is characterised by progressive hypoxaemia and alveolar infiltrates on a chest radiograph. PGD has significant impact on the short and longer term outcomes for lung transplant patients. Ex-vivo lung perfusion is one strategy to improve assessment of the donor organ and has the potential to act as a platform for implementing interventions to reduce the risk of or prevent PGD.

In 2005, the International Society for Heart and Lung Transplantation (ISHLT) published their standardised definition for PGD[52]. In this definition, the Pa02/Fi02 (P/F) ratio and the presence of bilateral infiltrates on a chest radiograph consistent with non-cardiogenic pulmonary oedema are assessed, **Table 1.3.** Assessment is carried out at specific time points after reperfusion; within the first 6 hours (T0), post

24 hours (T24), 48 hours (T48) and 72 hours (T72). Ideally, the P/F ratio is measured on a Fi02 of 1.0 and positive end expiratory pressure (PEEP) of 5 cm H20. The radiographic findings of PGD are non-specific and include peri-hilar ground glass opacities, peri-bronchial, perivascular thickening and reticular interstitial and airspace opacities located in a dependant fashion. Other contributory factors that can mimic, modify and confound the definition and grading need to be excluded, including cardiogenic oedema, pneumonia, hyper-acute rejection and pulmonary venous anastomotic obstruction.

PGD Stage	P/F ratio (mmHg)	Chest radiography	Updates from 2016 Consensus Group
0	>300	Normal	Any P/F ratio
1	>300	Diffuse allograft infiltration/Pulmonary Oedema	No changes
2	200-300	Diffuse allograft infiltration/Pulmonary Oedema	No changes
3	<200	Diffuse allograft infiltration/Pulmonary Oedema	No changes

Table 1.3 ISHLT PGD definition and severity grading 2005 and updates from 2016

A detailed review of the pathophysiology of primary graft dysfunction can be found in **Appendix A**: Morrison MI, Pither TL, Fisher AJ. Pathophysiology and classification of primary graft dysfunction after lung transplantation. *Journal of Thoracic Disease*. 2017;9(10):4084-4097. doi:10.21037/jtd.2017.09.09. Permission from the publishers, the AME Group has been granted. My contribution to this review article was in literature research, discussion and writing the manuscript.

The hallmark of PGD pathophysiology is the migration of polymorphonuclear neutrophils (PMNs) from the pulmonary circulation into interstitium and the airways. These PMNs are attracted out of the circulation by chemotactic mediators such as CXCL8 and damage-associated molecular patterns (DAMPs) which have been

released from apoptotic and necrotic lung tissue following ischaemia-reperfusion injury (IRI). Both HMGB1 and ATP are well-renowned DAMPs, thus IRI-mediated damage of the pulmonary airways serves to release these into the circulation and facilitate attraction of neutrophils to the lungs in the case of acute pulmonary diseases such as PGD.

Alongside neutrophil activation and migration, activation of pulmonary endothelium is another very important part of the pathophysiology of PGD. This activation occurs through the release of pro-inflammatory cytokines TNFα and IL-1β which enables up-regulation of adhesion molecules on the surface of the endothelium[53, 54]. E-Selectin interacts with P- and L-Selectin on the surfaces of activated endothelial cells to facilitate weak binding, as the latter roll along the endothelial surface through a cytokine ‘gradient’ before being arrested via integrin binding. Once localised to the lungs, transmigration of PMNs into the interstitial space and airways is facilitated by up-regulation of adhesion molecules, notably ICAM-1 and PECAM-1[54]. Support for the significance of the models is demonstrated by the study conducted by Simms et al, who identified heightened CD11b/CD18 cell surface expression as being associated with individuals who displayed acute respiratory distress syndrome (ARDS)[55]. Initiation of IRI provokes endothelial cells to up-regulate various adhesion markers on their surface, produce cytokines and begin actively contributing to inflammation[56-58]. ICAM-1 and PECAM-1 have both been implicated in acute lung injury and inflammation in general, with these facilitating leukocyte transmigration from the circulation into the airways[53].

The other major effect that stimulation of the endothelium causes is production of cytokines by these cells. Endothelial cells have been shown to directly participate in amplifying the inflammatory response and can produce PGD-associated cytokines. These are detrimental to the function of the lung, with levels of both of these examples having been linked with worsening outcomes of PGD in patients[59-61]. Activation of endothelial cells primes the environment for the secondary ‘wave’ of leukocyte influx into the pulmonary airways. It is this secondary phase that is ultimately so damaging. Whilst epithelial damage is an early hallmark of primary graft dysfunction evidence supports the contributory role that these cells in fact have towards worsening of pathology in many cases.

Lymphocytes are typically more involved in the latter stages of PGD development. Once the 'resident' alveolar macrophages have detected and responded to IRI within the lungs, they can then initiate intercellular communication with donor T lymphocytes. CD4+ cells mediate these effects in PGD, and to a much lesser extent CD8+ cells.

Use of alternative donor sources- DCD vs DBD

In the first clinical lung transplantation by Hardy[1], a DCD donor who died from myocardial infarction was utilised. At that time, use of DCD donors was a necessity as the concept of brain death was not yet legally established. Once brain death had reached the status of general acceptance in the 1970s, the majority of organs were retrieved from brain-dead donors with intact circulation. In the early nineties, Egan *et al*. carried out a number of experiments in dogs which caused a resurgent interest in the potential use of lungs from DCD donors. His group demonstrated that lung cells remain viable for a certain period after circulatory arrest[15]. The lung is the sole solid organ that is not dependent on perfusion for aerobic metabolism but rather uses a mechanism of passive diffusion through the alveoli for substrate delivery. Numerous experimental studies continued to investigate the possibility of using lungs from DCDs for transplantation[62, 63]. At the First International Workshop on DCDs in Maastricht in the Netherlands in 1995, five types of donors were identified, so-called Maastricht categories, **Table 1.4**.

I	Dead on arrival to hospital	Uncontrolled
II	Unsuccessful resuscitation	Uncontrolled
III	Awaiting cardiac arrest	Controlled
IV	Cardiac arrest following brain death	Controlled
V	Cardiac arrest in a hospital inpatient	Uncontrolled

Table 1.4 Maastricht categories of donation after cardiac death

Classification of donation after cardiac death (DCD). Categories I (dead on arrival) and II (unsuccessful resuscitation) comprise the uncontrolled donors. Categories III (awaiting cardiac arrest) and IV (cardiac arrest in brain-dead donor) include the controlled donors. A fifth category, V, cardiac arrest in a hospital inpatient, has recently been added[64].

Clinically, the first description of the use of lungs from DCD is from Love *et al.* in 1995 (n=3). This series, using lungs from category III donors, was updated in 2003 (n=20). In 2001, Steen reported successful lung transplantation using a category II DCD who died in hospital after failed resuscitation following myocardial infarction[65]. In 2004, the Madrid group published results from 2 successful lung transplantations from uncontrolled DCDs (Category I)[66]. This series has been updated more recently (n=17)[67].

The utilisation rates of DCD donor lungs have increased steadily over the last years and are comparable in early survival and lung function to standard DBD donors, as reported by Cypel[68]. Mason *et al*. compared survival after lung transplantations of controlled DCD versus DBD in the US using the UNOS data registry from October 1987 to May 2007[69]. During this period, 14,939 transplants were performed, including 36 (4%) with DCD organs (9 single, 27 double). Three of those died during the first 11 days. The 1, 6, 12, and 24- month patient survival was 94%, 94%, 94%, and 87%, respectively, for DCD donors versus 92%, 84%, 78%, and 69%, respectively for DBD (p=0.04). The authors concluded equivalent survival among DCD and DBD.

More recently, in May 2012, Mason *et al*. published a small retrospective single centre experience of DCD lung transplantation[70]. From August 2004 to July 2011, 605 patients underwent lung transplantation at the Cleveland Clinic, Cleveland, Ohio. 32 (4.9%) received a DCD lung transplant. The same standard donor selection, procurement, and preservation protocols for DBD were applied to DCD organs. Survival was 97% at 30 days, 91% at 1 and 2years, and 71% at 3 and 4 years. Mason *et al.* concluded that concerns over diminished organ quality are unfounded, and use of DCD lungs should be expanded. A meticulously performed meta-analysis of five studies found no difference in short or long-term survival, between transplants performed with DCD or DBD donors[71]. The International Society for Heart and Lung Transplantation Donation After Circulatory Death Registry Report published in 2015 demonstrated excellent survival after lung transplantation using DCD donors. This large study of international, multi-centre experience reported; thirty-day survival of 96% in the DCD group and 97% in the DBD group, one-year survival 89% in the DCD group and 88% in the DBD group and five-year survival of 61% in both groups[72].

Lung Preservation

In the current paradigm of lung transplantation, the decision to utilise an organ for transplantation is made at the time of organ retrieval. Once the decision to utilise a donor lung is made, the lungs must be procured from the donor at which point the obligate *ex vivo* phase begins. At the time of procurement, many strategies are employed in an attempt to better preserve the donor lung[18]. First, a lung protective strategy for ventilation is utilised during procurement to avoid further injury from barotrauma, and full anticoagulation of the donor (300 U Heparin/kg) is achieved to minimise the risk of intravascular clot formation.

Once the assessment of the donor is complete, aortic cross-clamp of the donor can commence. This arrests the heart and organ recovery can begin. A dose of 500 µg of prostaglandin E1(PGE1) is given into the pulmonary artery to lower the pulmonary vascular resistance by dilating the pulmonary vasculature, facilitating the subsequent flushing of the pulmonary vasculature. PGE1 has also been found to downregulate pro-inflammatory cytokine expression which may further help to reduce PGD[73].

The vasculature of the lung is then flushed to cool the lung tissue and to remove blood from the pulmonary vasculature, further minimising the potential for clot

formation and allowing for the removal of inflammatory and immune cells. The use of an extracellular type (i.e. low potassium) solution has been found to be beneficial to lung preservation as opposed to the intracellular type solution used in other organs[74]. Dextran 40 was also found to be a key ingredient in the lung flush solution and serves two purposes[75]. First, it acts as an oncotic agent to help keep fluid within the intravascular space. Second, it has the ability to reduce the aggregation of erythrocytes and thrombocytes, which preserves flow through the microvasculature following reperfusion, particularly in the bronchial microcirculation, and may play a role in reducing bronchial anastomotic complications. Another key ingredient in the flush solution is glucose. Because the lungs are stored inflated with oxygen, a unique situation arises during storage where the lungs are ischemic but not hypoxic. Glucose helps support aerobic metabolism in the lung during preservation. This flush solution is administered anterograde into the pulmonary artery and retrograde into each of the main pulmonary veins.

Approximately 50-60 ml/kg of perfusate total is utilised for anterograde and retrograde flushing. The desired flush pressure is a balance between too high a pressure leading to injury of the pulmonary vasculature and too low a pressure leading to inhomogeneous flushing. Use of low potassium dextran-glucose flush solution (LPD-glucose) has improved post-transplant outcomes. In a retrospective study by Oto *et al*., they showed that recipients of lungs stored with LPD-glucose had lower rates of PGD, days on ventilator, and 30-day mortality in comparison to other intracellular-type (high potassium) flush solutions[76].

Following the flush, the lungs are removed, inflated at an airway pressure of 20 cm H2O with 50% oxygen gas, triple-bagged, and stored on ice. Inflation of the lungs serves two purposes. First, it provides oxygen to the lung parenchyma for aerobic metabolism and secondly, it preserves the alveolar structure during storage; preventing collapse. Accordingly, Van Raemdonck *et al*. have shown that inflation even with nitrogen is still superior to atelectatic storage[77].

Once the lungs have been removed from the body, reduction of the metabolic rate by cooling of the lungs remains the cornerstone strategy for lung preservation today. Kayano *et al*. have shown in a rat model that the optimal temperature for lung preservation is approximately 10°C[78]. However, to simplify transport logistics, 4°C

is most commonly used. Once removed from the body, transplantation into the recipient should occur as soon as possible. PGD and 30-day mortality have been reported to increase with cold ischemic times longer than 8 h[79].

The history of ex-vivo lung perfusion

While the current cornerstone of clinical lung preservation has been to limit the metabolic rate by hypothermia, this strategy best serves lungs meeting ideal acceptance criteria. With the current donor organ shortage, most programs now utilize increasing numbers of extended criteria organs where lung function is not as assured as in ideal lungs. Ideally, further evaluation and even reconditioning of the lungs would be possible during the ex vivo phase of the organ before transplantation into the recipient. The limitation of the metabolic rate by static hypothermic preservation, precludes the possibility of meaningful lung evaluation and recovery. Preservation of donor organs would, therefore, need to occur at normothermic or sub-normothermic conditions to achieve these goals. One such strategy has been that of ex vivo lung perfusion (EVLP). This strategy attempts to simulate the in vivo situation by ventilation and perfusion of the donor lung graft. In 1935, Alexis Carrel and Charles Lindhbergh demonstrated that organs could remain viable for several days, reporting the first ex-vivo normothermic organ perfusion[80]. It would take until the 21st century ,however, for the first successful clinical EVLP to be reported when Steen *et al.* published their first case report in 2001, in Lund, Sweden[65]. They evaluated EVLP originally as a method of assessing lungs from an uncontrolled DCD donor prior to transplantation. They reported for the first time that lungs could be transplanted successfully after a period of warm ischaemia, EVLP, evaluation and cold storage. Steen retrieved lungs from a 54-year-old donor who had suffered a myocardial infarction and then had 190mins of cardiopulmonary resuscitation (CPR). Following this, EVLP was performed for 65mins, using the Lund protocol and resulted in a 54 –year old woman with COPD undergoing a single right lung transplant. The Lund group described their circuit which all future circuits would be based upon. The circuit included a pump generating a perfusate flow through an oxygenator, a leucocyte filter, a heater-cooler unit and gas exchange membrane before returning to the lungs through a cannula in the pulmonary artery. The pulmonary venous return was collected into a reservoir from the left atrium and then recirculated, **Figure 1.4**. Following this, several groups worldwide have investigated the role of EVLP in increasing the number of suitable pulmonary grafts and also in reducing the

incidence of primary and late graft dysfunction and overall lung transplantation outcomes.

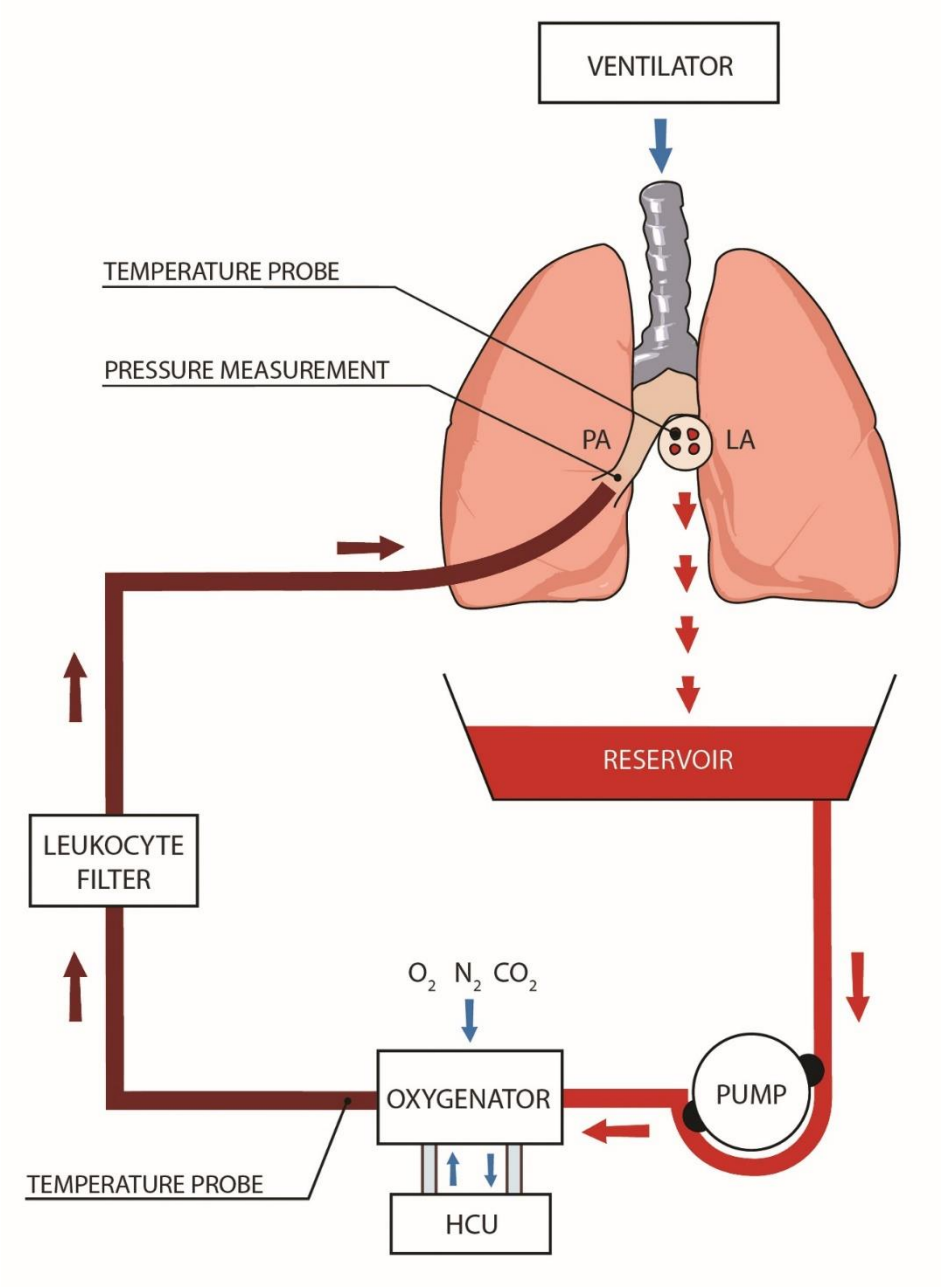

Figure 1.4 The EVLP circuit

Line diagram of an ex vivo lung perfusion (EVLP) circuit. Demonstrates the direction of flow of perfusate solution into the pulmonary artery (PA), through the lung vasculature, out from the left atrium (LA), collecting in a reservoir, through a pump, oxygenator, heater cooler unit (HCU), leucocyte filter and then returning to the PA. Adapted from Wallinder et al.[81]

Professor Stig Steen was the EVLP pioneer, who focussed primarily on the evaluation of uncontrolled DCD lungs. Dr Shaf Keshavjee, leading the Toronto group, concentrated on developing a functional EVLP model for donor lung perfusions over longer periods of time. The development of a stable EVLP model that would work over several hours would provide the basis for interventional, ex vivo treatment of the sub-optimal donor lung. In 2007 the Toronto group presented, at the ISHLT annual meeting in San Francisco, the first concept and feasibility of prolonged EVLP (12h) in association with an active treatment strategy (IL-10 gene therapy) to repair injured donor lungs ex vivo[82]. Subsequent publications, demonstrated the benefits of having a period of normothermic EVLP during the lung preservation process and a seminal prospective clinical trial demonstrated the feasibility and safety of EVLP to assess and improve the function of injured donor lungs or lungs from donors after cardiac death[27]. An explosion of EVLP research has since then occurred, and EVLP has emerged as one of the most promising recent advances in the field of lung transplantation and lung donor pool expansion.

Various international groups have extensively published evidence on the safety and effectiveness of EVLP as a way of assessment and reconditioning lungs deemed unsuitable for clinical transplantation. In more recent years, there has been a large body of work published locally in Newcastle by Andreasson *et al.* focussed on the immunological, inflammatory and tissue injury biomarkers during EVLP and the correlation to lung performance during assessment and after transplantation[83].

The next chapter in the journey of EVLP is to use the pre-existing knowledge we have and translate this into the use novel interventions that could potentially prevent reperfusion injury and ischaemia and modulate the immunogenicity of the donor lungs to prevent rejection. The current situation of lung transplantation is such that the decision to utilise donor lungs is made prior to explant and the possibility to repair the organ is limited to the donor prior to retrieval. With the development of EVLP, two new paradigms now exist. Firstly, evaluation of the donor lung and the decision to utilise the organ now can occur following retrieval and at the transplant hospital. Secondly, during the time the lung is on EVLP, treatments individualised to identified lung injuries can be applied; repairing acute injury associated with the donation process and salvaging these lungs for utilisation.

The rationale of EVLP

EVLP is a novel technique in which extended criteria donor lungs can be assessed objectively and potentially reconditioned for safe use in clinical lung transplantation; thereby safely increasing the donor pool. EVLP provides an alternative to the static cold preservation normally applied to organs in the period between removal from the donor and transplantation into the recipient. During EVLP, the lungs are not exposed to ischemia or the depressed metabolic state that hypothermia induces. The rationale for EVLP under physiologic conditions is to allow pulmonary tissue to remain metabolically active and viable for several hours prior to transplantation. This period during EVLP provides the opportunity for: prolonged lung preservation; assessment of the donor lungs and the re-conditioning of the organs from any damage associated with death and hypothermic storage. Ways in which the lungs could be reconditioned include but are not limited to: reduced oedema in lung tissue by the high oncotic pressure of the perfusate; removal of harmful and toxic waste products with filters and membranes within the circuit and recruitment of atelectatic areas resulting in improved ventilation/perfusion mismatch. There is also great potential for delivering therapies to the pulmonary graft and investigating and developing our understanding of the complex inflammatory and immune-mediated pathways and mechanisms involved in the ischaemic-reperfusion process.

The EVLP technique and available devices

Irrespective of the EVLP protocol or device used, the premise of EVLP always remains the same. The equipment always includes a pump generating a perfusate flow through an oxygenator, a leucocyte filter, connected to a heater-cooler unit and gas exchange membrane before returning to the lungs through a cannula in the pulmonary artery. Pulmonary venous return is collected into a reservoir from the left atrium and then recirculated. A ventilator is connected to the trachea allowing “protective” ventilation to be carefully started only after the lungs have been rewarmed to 32^{o}C and steadily increased whilst approaching normothermia. Irrespective of the EVLP protocol used the procedure has distinct phases. The EVLP procedure, can be described in the following phases:

Preparation

The EVLP circuit is primed with 2 litres (L) of perfusate solution, most commonly Steen Solution™ and additive medications such as heparin, antibiotics, antifungals and pH buffer. When the donor lungs arrive at the operating theatre of the receiving

centre, they must be prepared for the EVLP procedure. Any superfluous tissue is trimmed off, and the lungs are weighed. An endo-tracheal tube is secured into the trachea, which may have been shortened. A cannula is secured into the pulmonary artery; this will vary depending on the specific perfusion machine being used. If the donated heart is favoured and the pulmonary artery is cut at or after the bifurcation, the artery can be elongated with a graft, consisting of a piece of the donor aorta or a synthetic graft. Once the cannulations are complete, a retrograde flush is performed using Perfadex. The lungs are connected to the EVLP unit, and a temperature probe is sutured to the lumen of the left atrial remnant to record the temperature of the perfusate leaving the lungs. Perfusion starts with a slow antegrade flow to de-air the circuit and the lung vasculature.

Warming and reconditioning

After the lungs are connected to the EVLP unit, they are gradually rewarmed to body temperature. Bronchoscopy is performed for airway control and cleaning. At 32°C, ventilation is initiated with small tidal volumes and at low pressure. A protective ventilation strategy is adopted, similar to that adopted in treating Acute Respiratory Distress Syndrome (ARDS) patients in the Intensive Care Unit (ICU)[84]. The protective strategy involves using a 7 ml/kg tidal volume, a PEEP of 5 cm H20, a respiratory rate of 7 breaths per minute and an oxygen concentration of 21 %. Since the warming of the perfusate takes place with a pre-set difference in temperature of 8°C between the in- and out-flowing blood, the duration of the warming is dependent upon flow and the size of the lungs. Once the lungs begin ventilation, a gas mixture (86% N2,8% CO2, 6% 02) is turned on and applied at a sweep at 1 litre/min. A step-wise increase in the perfusion flow is implemented. The perfusion flow rate is dependent on which protocol is being used, and this will be discussed shortly.

Evaluation

Once a temperature of 36°C is reached, the evaluation phase is started. At this point, the O_2 supplied from the EVLP unit is discontinued. In this phase, the oxygenator is only used for deoxygenation and the provision of CO_2, to mimic the body. Oxygenation and removal of CO_2 from the perfusate are now dependent upon lung function, which can be evaluated by analysing the blood gases. The lung compliance, dead space fraction, respiratory pressures and peripheral vascular resistance (PVR) are evaluated repeatedly during this phase.

Cooling and storage

If the lungs are deemed transplantable, the oxygenator is once again used as an O2 provider. The lungs are rapidly cooled to 12°C, with ventilation terminated at 32°C.

Several commercial companies have designed and marketed devices for clinical EVLP, **Figure 1.5**:

- Organ Care System (OCS) Lung (TransMedics)
- Vivoline LS1 (Vivoline Medical, Lund, Sweden) Vivoline has now been taken over by XVIVO.
- Lung Assist (Organ Assist, Groningen)
- XPS (XVIVO Perfusion AB)
- XOR Labs Toronto

Figure 1.5 Commercially available EVLP machines

Semi-automated EVLP circuits used in ongoing multicentre trials investigating EVLP in clinical lung transplantation. (A) Vivoline® LS1, (Vivoline Medical AB), static EVLP with the Lund EVLP protocol. (B) XPS™, (XVIVO Perfusion AB), static EVLP with the Toronto EVLP protocol. (C) OCS™ Lung, (TransMedics Inc.), portable EVLP with the OCS EVLP protocol. Andreasson et al. [85]

EVLP DEVICE	DEFINING DESCRIPTORS
OCS Lung (Trans Medics, US)	• Portable • Contains battery power, gas cylinders and monitoring equipment. • Pump has a pulsatile-type flow • Potential for eliminating long periods of cold ischaemia
Vivoline (Lund, Sweden)	• Pump has an internal roller to create continuous flow • Requires an external ventilator and gas cylinders to commence EVLP.
Lung Assist (Groningen, Netherlands)	• Less robust • Individual components mounted on a frame
XPS (XVIVO Perfusion AB)	• Fully integrated device • Centrifugal pump generating continuous flow. • X-ray possibilities

Table 1.5 Comparison between commercially available EVLP devices.

Comparison of the commercially available EVLP devices and there unique selling points.

EVLP protocols

Three EVLP protocols have been well examined in clinical trials: Toronto, Lund and Organ Care Systems. The Lund protocol, designed by Steen *et al*. was primarily designed for a shorter perfusion period and for the evaluation of initially rejected donor lungs and uncontrolled DCD lungs. The Lund group EVLP circuit provided the blueprint for all circuits currently used in clinical centres worldwide. The Lund EVLP protocol is designed to mimic the physiologic conditions a transplanted lung will go on to face after reperfusion in the recipient. It serves primarily as a thorough assessment of borderline lungs not meeting standard acceptance criteria to reclaim those demonstrating suitability for clinical transplantation on the *ex-vivo* circuit.

The Toronto protocol focused on the feasibility of prolonged EVLP to allow for an extended period of assessment, reconditioning and to the development of future treatments to be applied during extended lung perfusion.

One difference between these methods is that a cellular evaluation solution is used in the Lund method. Also, in the Toronto method, the left atrium has a cuff attached, to allow a positive left atrial pressure during the perfusion, and in the Lund method, it is open. In the Lund method, 100% of the donor's estimated cardiac output is circulated through the lungs, as compared to 40% in the Toronto method.

In 2012, a third EVLP strategy was proposed, in which a portable EVLP unit allowed for perfusion to be instituted at the donor hospital and maintained during transportation and up until the transplantation. In a pilot study published in the Lancet 2012, Warnecke *et al*. investigated the effect of normothermic preservation and transportation of standard criteria human donor lungs on a portable EVLP system[86]. Twelve pairs of standard criteria human donor lungs were, instead of transported with cold static preservation on ice, preserved by normothermic perfusion and ventilation on the transportable Organ Care System (OCS) Lung (TransMedics, Inc., Andover, MA, USA). This pioneering report of a portable EVLP system reported non-inferiority in short-term outcomes to controls.

The Toronto method introduced the concept of extended EVLP (>12h) using an optimal lung protective strategy, aimed to avoid circuit-induced injuries due to mechanical shear stress and preserving cell homeostasis. There are three main differences between the Toronto method and other methods: 1) the use of an acellular perfusate, 2) a closed circuit with a positive left atrial pressure and 3) a lower perfusion flow rate. The defining differences between the three most commonly recognised EVLP protocols are outlined in **Table 1.6**. The acceptable variables that indicate lungs are suitable for transplantation at the end of EVLP are found in **Table 1.7.**

	TORONTO	LUND	ORGAN CARE SYSTEM
Application	Re-evaluation, reconditioning, treatment	Re-evaluation, reconditioning	Replace cold ischaemia
Transportable	No	No	Yes
Perfusate	Steen solution	Steen solution	OCS solution or Perfadex
Red blood cells added	NO - Acellular	YES - Cellular. RBC's haematocrit 14%	YES – Cellular. RBC's haematocrit 15-25%
PRESSURE			
Pump	Centrifugal	Roller	Piston (pulsatile)
Target Flow	40% of cardiac output	100% of cardiac output	2-2.5 l/min
Target PAP	<15mm Hg flow dictated	< 20 mm Hg	<20 mm Hg
Left atrium	Closed (3-5mmHg)	Open (0 mmHg)	Open(0mmHg)
VENTILATION			
Gas mixture	8% CO2, 6% 02, 86% N2	7% CO2, 03% N2	
Start Temp	32°C	32°C	34°C
Tidal volume	7 ml/kg IBW	3-8 ml/kg IBW	6 ml/kg IBW
RR	7	20	10
PEEP	5 cm H20	5 cm H20	5-7 cm H20
Fi02 (%)	21	50	21
TEMPERATURE (°C)	**TORONTO**	**LUND**	**ORGAN CARE SYSTEM**
Start perfusion	25	15	32
Start ventilation	32	32	34
Start evaluation	37	37	37
Time to stability	1h	1h	10-15mins
Total time perfusion	4-6 h (Up to 12 hr)	2-7 h (92-393min)	Transport time (188 - 622min)

Table 1.6 Comparison of EVLP protocols in clinical lung transplantation

Direct comparisons of the three most commonly used EVLP protocols in relation to their haemodynamic and ventilatory settings. IBW (Ideal body weight), MIN (Minutes), PAP (Pulmonary artery pressure), RBCs (Red blood cells), RR (Respiratory rate), PEEP (Positive End-Expiratory Pressure), FiO_2 (Inspired oxygen concentration), PO_2/FiO_2 ratio (Difference between inspired oxygen concentration and PO_2).

	TORONTO	LUND	ORGAN CARE SYSTEM
Transplant acceptable variables	PO2/Fi02 > 400mmHg Stable: PA pressure, PVR, airway pressures, lung compliance Clear chest x-ray Bronchoscopy	PO2/Fi02 > 300mmHg Stable: PA pressure, PVR, airway pressures, lung compliance Absence of consolidated lung collapse test	PO2/Fi02 ratio When system stable at donor site and before transplantation at recipient site

Table 1.7 Transplant acceptable variables at end of EVLP

Table showing the acceptable variables that must be met in order for lungs to be deemed acceptable for transplantation after EVLP. PO_2/FiO_2 ratio (Difference between inspired oxygen concentration and PO_2), PA (Pulmonary artery), PVR (Pulmonary vascular resistance).

Open versus closed circuit

EVLP systems use either an open or a closed circuit. In an open system, the heart is removed before the lungs are connected to the circuit and the remnant of the left atrium is opened to the atmosphere, leaving an atrial cuff. The alternative to this, a closed system involves a specially designed plastic cannula being sutured into the remnant of the left atrium, allowing maintenance of a positive left atrial pressure by adjusting the height of the reservoir. A distinctive feature of the Toronto EVLP protocol is the use of a closed left atrium at a pressure of 3-5 mmHg whereas the left atrium is open in the Lund protocol. In 2014, Linacre *et al.* published a comparative study on pig lungs to evaluate the relative effects of open or closed left atrium[87]. They reported a significant advantage for the closed atrium alternative when EVLP exceeded 4 – 5 hours. The full 12 hours of EVLP could not be completed in 4 of 5 open atrium cases due to pulmonary oedema: after 7 hours EVLP these lungs showed increases in PVR, peak inspiratory pressure and decreases in lung compliance and oxygenation. All lungs with closed atrium completed 12 hours EVLP.

Advocates of the closed method argue that by achieving left atrial pressures of 3-5 mmHg, you can counteract oedema forming by preventing the collapse of pulmonary veins and alveolar wall capillaries, during ventilation and maintaining venous afterload and microvascular recruitment, thereby reducing vascular resistance. It has been argued, however, that although with an open system, there is the possibility of venous collapse, that the actual risk is low when a fountain of perfusate with a height of 2–5 cm can be observed in left atrium throughout the EVLP procedure. Venous collapse should not occur as long as this phenomenon is observed. When using an open method, attention needs to be taken whilst dissecting off the heart. One must remove as much tissue as possible, to avoid any obstruction to outflow from the left atrium. In addition, an open atrium provides the opportunity to collect blood gases from selected pulmonary veins, thereby allowing evaluations of the functions of isolated parts of the lung.

Cellular versus acellular perfusate

One important question in designing any EVLP study is to decide on using either a cellular or acellular perfusate. The Lund and OCS protocols use a cellular perfusate, whereas the Toronto method employs an acellular solution. EVLP logistics are simplified and costs reduced in an acellular technique. Uniquely, oxygen supply to lung cells during EVLP appears to be primarily provided by the ventilator alone without the need of haemoglobin as an oxygen carrier[88]. During perfusion with a cellular perfusate solution, there is the potential risk of haemolysis due to mechanical trauma to the red blood cells. During the cold portion of perfusion, low temperatures may cause a significant increase in viscosity with cellular perfusates, which may impact on mechanical stress and additionally lead to haemolysis[88].

Conversely, Wallinder *et al.* reported good short-term outcomes after human transplantation with lungs assessed using a cellular perfusate for up to 9 hours[81]. A potential benefit of a cellular perfusate is the oxygen-binding capacity that erythrocytes provide and its possible advantage during lung evaluation. It is also argued that the presence of red blood cells provides a more physiologically relevant assessment of flow through the pulmonary microvasculature. Yeung *et al*. in a small animal model study demonstrated that blood gas analyses during EVLP with an acellular perfusate are unreliable and could disguise oxygenation deficits during assessment; especially in the case of a ventilation–perfusion mismatch[89]. The

shunting effect became obvious first when erythrocytes were added to the perfusate, and then vanished again when the perfusate was replaced with fresh acellular Steen Solution™. It has also been argued that a cellular perfusate is more physiologically representative. Using an acellular solution for the warming phase and then adding red blood cells for the remainder of the perfusion could be a possible compromise.

Roman *et al.* demonstrated no significant difference in the physiologic, immunologic, or ultrastructural parameters between lungs perfused with cellular or acellular solutions[90]. Becker et al also demonstrated that prolonged acellular and cellular EVLP for 12 h are both feasible with lungs pre-challenged by ischaemic organ stress. They found that the physiological and ultrastructural analysis showed no superiority of either acellular or cellular perfusate composition[91].

Low versus high flow

During EVLP, the intrinsic pulmonary vascular resistance (PVR) often regulates the flow. This is especially true during the re-warming phase of EVLP when the flow is always limited by the high PVR in the cold and rigid lung. The approaches taken towards flow rates, by the Toronto and Lund groups are different. Cypel *et al.* and the Toronto group have advocated for a maximum EVLP flow rate of approximately 40% of the estimated cardiac output[88]. Low flow has been argued to protect the pulmonary microvasculature against mechanical injury of the endothelium, maintaining the integrity of the alveolar-capillary barrier and preventing the effusion of fluid from blood vessels, thereby decreasing oedema formation. The Toronto group relies on an EVLP technique with low Pulmonary artery (PA) flow (40% of the estimated cardiac output), low PA pressure (PAP; 10–15 mmHg), and advocates a careful perfusion strategy with initially very low PA pressures. A low flow strategy is thought to facilitate perfusion over longer time periods. The low flow approach has the potential to avoid haemodynamic stresses due to the reperfusion of a previously hypothermic lung. Those advocating a low flow approach stress that it is vital to monitor mean PAP and aim to keep this below 15-20mmHg to avoid the development of hydrostatic pulmonary oedema.

The theoretical disadvantage with the low-flow regime is that a low pressure level could be insufficient for perfusion of the non-dependent parts of the lung, e.g., West zone 1. In an uncontrolled DCD situation with a lung that has impaired peripheral

circulation, the use of too careful of a perfusion regime may result in only a portion of the lungs being perfused. There is also concern that a reduced flow approach is less representative of the post-transplant load on the lung circulation.

In contrast, the full-flow evaluation with the Lund protocol uses a pre-set pressure limit of 20 mmHg from the start to the end of the perfusion. Thus, the flow is more dependent upon the PVR. A high flow approach aims to test lung function during EVLP, and simulate the conditions under which the lungs will subsequently be required to function. This may provide the assessor confidence in making a quick prediction of the organ's transplant suitability.

Perfusate solutions

The perfusate used in the EVLP circuit must meet some basic criteria. The colloid osmotic, or oncotic pressure, must be equal to or higher than the oncotic pressure in the blood. An oncotic pressure of about 25 mmHg exists typically in the capillaries. The hydrostatic pressure in the arterial end of the capillaries is normally about 30 mmHg, and the hydrostatic pressure in the venous end of the capillaries is approximately 10-15 mmHg. This pressure gradient causes water to leave at the arterial end and return at the venous end of the capillaries.

The commercially available solution that is currently used almost exclusively for EVLP in both laboratory and clinical settings is Steen Solution™ (XVIVO Perfusion AB). As the name implies, Stig Steen and colleagues developed this solution. This fluid has a high oncotic pressure (25–30 mmHg), which the manufacturer described as optimal for mobilisation of interstitial oedema often found in donor lungs. Steen Solution™ is an extra-cellular electrolyte composition (low K^+) with the addition of human albumin to maintain optimal colloid pressure and dextran 40, a mild ROS scavenger which coats and protects endothelium from subsequent excessive platelet/leucocyte interaction, complement, cell-mediated injury and to inhibit coagulation and platelet aggregation thrombogenesis.

In the Lund model, the EVLP procedure is performed with a mixture of Steen solution and washed erythrocytes to reach a haematocrit of 15% whereas, in the Toronto protocol, the EVLP procedure is carried out with an "acellular" Steen Solution™. In

the OCS model, OCS solution is used, made by the manufacturer and essentially a low potassium dextran solution with added glucose.

In an attempt to produce an alternative perfusate solution for EVLP, Fernandes *et al.* in a Brazilian study of 16 EVLPs compared Steen solution with an alternative solution made by a Brazilian pharmaceutical company[92]. From 16 lungs tested, they found no difference on weight after EVLP: Steen group (SG) = 1,097±526g; Alternative Perfusion Solution (APS) = 743±248g, p=0.163. Oedema formation, assessed by Wet/dry weigh ratio, was however, statistically higher on the Alternative Perfusion Solution group (APS = 3.63 ± 1.26; SG = 2.06 ± 0.28; p = 0.009). Steen Solution™ remains the main reconditioning solution for EVLP used both in clinical and experimental fields.

EVLP assessment and parameters

EVLP allows for a period of assessing donor lungs and provides more information for decision making. Oxygenation capacity is traditionally considered to be the most crucial parameter for the evaluation of donor lung function. During EVLP, blood gases are drawn from the oxygenated blood in the left atrium. The gas exchange in the evaluated lung at a certain fraction of inspired oxygen is dependent upon the following factors: a) the ventilation of the alveoli; b) the diffusion capacity of the blood-gas barrier; c) the amount of shunted blood; d) the ventilation/perfusion relationship. The prognostic value of the oxygenation capacity of the lung during EVLP has been questioned by the Toronto group[89]. Gradually decreasing dynamic compliance over time is instead suggested as the best indicator of inferior lung function.

In both animal and human EVLP, irrespective of the EVLP protocol employed, the PVR is higher than what would be expected for normal in vivo physiology[27]. This may be explained in part by the difference between the physiologic flow in vivo and the artificial flow generated by the pump in the EVLP unit.

The compliance of the lung reflects the change in lung volume for an applied pressure. Static compliance is calculated during an inspiratory hold manoeuvre (static condition). Compliance is calculated as the inspired tidal volume is divided by the insufflation pressure at the end of inspiration (plateau pressure) minus the

positive end-expiratory pressure. Compliance, and especially changes in compliance over time, has been suggested as important parameters for evaluating donor lung function during EVLP[89].

Despite being the organ with the highest oxygen concentration in the body, about 40% of the glucose that is metabolised in the lung becomes lactate. As the EVLP circuit is closed and the lungs' ability to consume lactate is limited, the lactate level in the lung increases during EVLP. The level of lactate during EVLP has however been found to be unrelated to the function of the perfused lung[93].

A high rate of glucose consumption during EVLP has been suggested as a marker of graft quality. In a group of animals with high glucose consumption, pulmonary oedema was more pronounced than in the controls. However, gas exchange capacity and hemodynamic parameters showed no association with the rate of glucose consumption[94].

Oedema of the donor lung is a frequent cause of impaired function; and in the laboratory setting, a resected part of the lung can be weighed and then dried in an oven until no further weight loss is recorded. The wet to dry weight ratio is then a measure of tissue oedema and is often demonstrated in studies. The parameters which can be assessed during EVLP are summarised in **Table 1.8.**

ASSESSMENT	PARAMETER
HAEMODYNAMIC	• Pulmonary artery pressure (PAP) mmHg) • Peripheral vascular resistance (PVR) • Flow rate (RPM)
VENTILATORY	• Compliance • Peak Airway Pressure • Visual inspection of consolidation and atelectasis
OXYGENATION	• Pa02/Fi02 ratio • Arterial blood gases: $p0_2$, $PC0_2$
BACTERIAL LOAD	• Bronchoalveolar lavage (BAL): gram stain and culture
LUNG OEDEMA FORMATION	• Wet/Dry ratio • Chest radiograph (CXR) • Visual inspection of pulmonary oedema
INFLAMMATORY RESPONSE	• Cytokine analysis

Table 1.8 Summary of the parameters that can be measured, monitored and assessed during EVLP.

EVLP Clinical Trials

Several prospective studies of various EVLP techniques have been completed in Europe and North America. A summary of recent clinical trials is given in **Table 1.9**. These include the HELP trial (Toronto; sponsor XVIVO Perfusion), the NOVEL trial (USA; sponsor XVIVO Perfusion), the INSPIRE and EXPAND trial (Europe and USA; sponsor TransMedics), the Develop UK (UK; sponsor Vivoline), the Vienna trial (Vienna, sponsor XVIVO), and the Perfusix Trial (USA; sponsor Perfusix).

Name of clinical trial	Centre	Primary endpoint	Sponsor	Method
HELP	Toronto (Cypel et al) 2011	PGD scores in the first 72 hrs, 30 day mortality	Vitrolife	Toronto
NOVEL	Multicentre (US)	30 day mortality	XVIVO	US
DEVELOP	UK	12-month survival Cost effectiveness	Vivoline	Lund
EXPAND	Multicentre (International)	PGD scores in the first 72 hrs, 30 day mortality	Transmedics	OCS
INSPIRE	Multicentre (International)	PGD scores in the first 72 hrs, 30 day mortality	Transmedics	OCS

Table 1.9 Summary Table of Recent EVLP Trials

HELP Trial

The Toronto EVLP method represents arguably the most extensive clinical experience of EVLP. The Toronto General Hospital Transplant Program EVLP experience has been routinely published and updated every year. In 2011, Cypel *et al.* published their experience from September 2008 through January 2010[27]. During the study period, 23 donors met the inclusion criteria that defined potential donors as “high risk” for not having acceptable lungs. Those 23 donor lungs underwent EVLP. One hundred and sixteen standard lungs transplanted during the same period preserved with static cold storage constituted the control group. The primary endpoint was PGD of Grade 2 (PaO2/FiO2 of 200 to 300 mm Hg) or Grade 3 (PaO2/FiO2 <200 mm Hg), according to ISHLT[95]. Secondary endpoints were 30-

day mortality, bronchial complications, duration of mechanical ventilation, and length of stay in the intensive care unit (ICU) and hospital. Out of 23 high-risk donors who underwent EVLP, 20 were deemed appropriate for transplantation. The lungs were transferred to Toronto General Hospital according to standard practice and placed in the EVLP system (XVIVO™, Vitrolife) and perfused at 37°C with STEEN Solution™ (Vitrolife, Inc.) for four hours. After four hours of EVLP, the donor lungs were evaluated for delta PO2 ≥ 350 mm Hg and stable pulmonary vascular resistance (PVR), peak airway pressure (PawP), and lung compliance. Criteria regarding the termination of perfusion and non-transplantability consisted of a PVR, dynamic compliance, peak inspiratory pressure decline by more than 15%, and FiO2 less than 350 mmHg. By comparing the EVLP group to contemporary transplants utilising standard criteria organs, no noteworthy dissimilarities could be found with respect to PGD rates, days of mechanical ventilation after transplant, lengths of ICU and hospital stays or 30-day mortality.

In 20 of 23 EVLP lungs, the physiological function remained stable during EVLP and the median PaO2/FiO2 ratio increased from 335 mm Hg in the donor lung to 414 and 443 mm Hg at 1 hour and 4 hours of perfusion, respectively ($p<0.001$). The incidence of PGD Grades 2 and 3 at 72 hours after transplantation was 15% in the EVLP group and 30% in the control group ($p=0.11$). Severe PGD (Grade 3) in the EVLP group was 0%, and 9.4% in the control group ($p=0.36$). No significant differences were observed for any secondary endpoints, and the 1-year post transplant survival rate was 80% for EVLP lungs and 83.6% for control lungs ($p=0.54$). They concluded that 4 hours of EVLP led to immediate and one-year post-transplant outcomes similar to those obtained with conventionally selected lungs

The latest update of the Toronto General Hospital EVLP Transplant Program experience was published by Cypel *et al.* in 2012[96]. This update included lung transplants with high risk DBD and DCD donor lungs. During the study period of 39 months, a total of 317 lung transplants were carried out. 58 EVLP procedures, durations of between 4-6 hours, were performed in high risk DBD and DCD donor lungs, resulting in 50 transplants (86% use). Lungs that achieved stable airway and vascular pressures and PaO2 /FiO2 greater than 400 mm Hg during ex vivo lung perfusion were transplanted.

The incidence of PGD Grade 3 at 72 hours was 2% in the EVLP group and 8.5% in the control group (p=0.14). Thirty-day mortality (4% in the EVLP group and 3.5% in the control group, p=1.00) and 1-year survival (87% in the EVLP group and 86% in the control group, p=1.00) were similar in both groups. The authors concluded that transplantation of high-risk donor lungs after 4 to 6 hours of EVLP is safe, and outcomes are similar to those of conventional transplants. Prior to the use of EVLP, Toronto General Hospital used approximately 25% of available donor lungs. After initiating the HELP study and using EVLP lungs, the overall lung utilisation rate increased to 36%.

NOVEL Trial

The NOVEL trial was a prospective, controlled, non-randomized, multicentre (6 US centres), open label, non-inferiority study; including patients transplanted with "extended criteria lungs," (as defined by the inclusion criteria for EVLP), compared to standard lung transplant recipients[97]. The primary study endpoint was 30-day mortality. The secondary study endpoints included 72-hour PGD, length of ICU stay and ventilator or extra-corporeal membrane oxygenator (ECMO) usage. During the period of May 2011 to May 2014, 54 lung donors met the criteria and underwent EVLP, using the Toronto method and XPS system. From this group, 29 (54%) were transplanted into 31 recipients. 15 recipients received double lung transplants and 16 recipients received single lung transplants, with 12 single lungs discarded. Additionally, 25 donor lung pairs (46%) were discarded after EVLP and not transplanted. For the control group, 62 standard criteria lungs were transplanted into 31 recipients, with 19 (61%) receiving double lung transplants and 12 (39%) receiving single lung transplants. At the end of EVLP, the median PaO2/FiO2 was 500 and 375 mm Hg in the in the EVLP transplanted and EVLP-non-transplanted groups, respectively. 30-day survival was 97% vs.100% in the EVLP and control arms, respectively. 1-year survival was comparable across EVLP (87%) and control group (93%). The incidence of PGD Grade 3 at 72 hours was 10% and 3% in the EVLP and control groups, respectively.

DEVELOP- UK

The objective of the Donor Ex Vivo Lung Perfusion in UK lung transplantation: DEVELOP-UK study was to evaluate the clinical and cost-effectiveness of EVLP towards increasing UK lung transplant activity[98]. It was a multicentre, un-blinded,

non-randomised, non-inferiority observational study to compare transplant outcomes between EVLP-assessed and standard donor lungs, involving all five UK officially designated National Health Service (NHS) adult lung transplant centres. Patients aged ≥18 years with advanced lung disease accepted onto the lung transplant waiting list could participate. The primary outcome measure was survival during the first 12 months following lung transplantation. Secondary outcomes were patient-centred metrics, influenced by the effectiveness of lung transplantation and that contribute to the overall health-care costs. Lungs from 53 donors found unsuitable for standard transplant were assessed with EVLP, of which 18 (34%) were subsequently transplanted. A total of 184 participants received standard donor lungs. Patients in the EVLP arm required ventilation for a longer period and stayed longer in an ICU than patients in the standard arm, but the duration of overall hospital stay was similar in both groups. There was a higher rate of very early grade 3 PGD in the EVLP arm, but rates of PGD did not differ between groups after 72 hours. The requirement for ECMO support was higher in the EVLP arm (7/18, 38.8%) than in the standard arm (6/184, 3.2%). The Kaplan–Meier estimate of survival at 12 months was 0.67 [95% confidence interval (CI) 0.40 to 0.83] for the EVLP arm and 0.80 (95% CI 0.74 to 0.85) for the standard arm. There were no significant differences in rates of chest radiograph abnormalities, infection, lung function or rejection by 12 months. The cost of EVLP transplants was found to be approximately £35,000 higher than the cost of standard transplants, as a result of the cost of the EVLP procedure, and the increased ECMO use and ICU stay. The study had important limitations including small numbers in the EVLP arm due to the early study termination, limiting the analysis to descriptive statistics and the EVLP protocol change during the study. The reason for the increased PGD rates, ECMO requirement and possible differences in lung injury between EVLP protocols all need further evaluation.

INSPIRE

The INSPIRE trial aimed to assess the impact of ischaemia on short and long term outcomes in standard criteria double lung transplants[99]. The trial involves 21 centres across Europe and the United States and aims to compare standard cold storage versus preservation by the portable OCS Lung perfusion and ventilation system in standard criteria donor lungs. The primary end-points were 30-day survival and severe PGD-3 at 72 hours. Those in the OCS arm had significantly shorter periods of cold ischaemic time, and this has been shown to be the most significant

predictor for developing PGD-3 within 72 hours. The authors also concluded that there was a strong correlation between ischaemic time and the development of BOS at 2 years' post-transplantation.

EXPAND

The OCS Lung EXPAND trial was a prospective, multi-centre, international trial to assess the short and one year clinical outcomes after lung transplantation of Extended Criteria Donor (ECD) lungs that were preserved and assessed using the OCS portable lung perfusion and ventilation system. The results of the this trial were presented at the ISHLT 38th Annual Meeting in Nice, April 2018[100]. The inclusion criteria included double lung donors that met any of the following criteria: age > 55 years, P/F ratio less or equal to 300mmHg, expected ischaemic time >6hours or DCD donation. Seventy-nine out of 91 eligible donor lungs were successfully transplanted, a utilisation rate of 87%. The incidence of PGD-3 at 72 hours was 6.4%. 30-day, 6-month and 1-year survival rates were 99%, 93% and 91% respectively.

EVLP as platform for assessment and treatment

Recent work has focussed on targeting the inflammatory and immunological pathways involved in ischaemic-reperfusion injury and several studies have investigated the main culprit pathways involved in this process. EVLP has been used as a platform for not only assessing the physiology of the donor lung but also the inflammatory and immunological status of the organ.

Krishnadasan *et al.* investigated the role of TNF- α and IL-1β in regulating the development of lung ischaemia-reperfusion injury[101]. They appear to promote injury by altering the expression of pro- and anti-inflammatory cytokines and influencing tissue neutrophil recruitment. Animals receiving anti-tumour necrosis factor- α and anti-interleukin-1β demonstrated reduced injury compared with that seen in positive control animals (vascular permeability of 48.7% and 29.4% lower, respectively; ($p < .001$). Vascular injury was reduced by 71% when antibodies to TNF-α and IL-1β were administered together. Lung neutrophil accumulation was markedly reduced among animals receiving anti- TNF- α and anti- IL-1β. BAL leukocyte content was also reduced by treatment with, anti- TNF- α and anti- IL-1β and combination treatment. Reductions in permeability, myeloperoxidase, and BAL leukocyte content also resulted in a decrease in lung injury assessed histologically. Finally, anti- TNF- α and anti- IL-1β treatment resulted in decreased messenger RNA expression for many early response and regulatory cytokines.

Following brain death, a systemic inflammatory response known as a 'cytokine storm' transpires. Increased circulating pro-inflammatory cytokines results in the induction of cell adhesion molecules on pulmonary endothelial and epithelial surfaces and leads to the recruitment of neutrophils and monocytes to the lung causing inflammatory lung injury.

Fisher *et al.* studied the levels of IL-8 in BAL fluid from 26 donor lungs used for transplantation and showed that a high concentration of IL-8 in donor BAL was correlated with severe graft dysfunction and with early postoperative deaths[43].

Similarly, De Perrot *et al.* showed that interleukin IL-8 levels in donor lung tissue before and after transplantation increased with time after reperfusion and that

patients who developed severe primary graft dysfunction had significantly higher IL-8 levels during ischaemia and after reperfusion[42].

Andreasson *et al.* have recently investigated the role of interleukin-1β as a predictive biomarker and potential therapeutic target during clinical ex-vivo lung perfusion, analysing EVLP samples from DEVELOP-UK[83]. The protein markers with the ability to differentiate declined lungs from the survival group after 30 minutes of perfusion were IL-1β and TNF-α in perfusate, p=0.004 and p=0.001 respectively. The most effective markers to differentiate recipient in-hospital mortality (non-survival group) from those successfully discharged (survival group) were similarly perfusate IL-1β p<0.001 and TNF-α p=0.003 after 30 min of perfusion. With a TLC scaled cut-off value of 0.1 pg/ml, perfusate IL-1β after 30min of EVLP had a sensitivity and specificity of 100% to diagnose non-survival lungs. The predictive value of perfusate IL-1β and TNF-α after 30 min of EVLP remained robust when applied to the primary study endpoint of 1-year post-transplant survival. With a TLC scaled cut-off value of 0.1 pg/ml, perfusate IL-1β after 30min of EVLP had a diagnostic sensitivity of 83% and specificity of 100% for recipient 1-year post-transplant mortality.

EVLP studies worldwide are investigating the potential for EVLP as a platform for lung reconditioning and repair prior to implantation. There are several target areas for novel interventions and therapies that can be delivered during EVLP; namely reducing oedema and endothelial leak, inflammation, infection and thrombi.

Andreasson *et al.*[102], demonstrated the potential EVLP holds by showing the therapeutic impact of administering broad-spectrum antibiotics added to the perfusate and circuit during EVLP. In this study, 18 human donors deemed unsuitable for transplantation that underwent EVLP were examined. 13 out of 18 lungs had positive cultures commencing EVLP with bacterial loads significantly decreased after EVLP. Yeast loads reduced when prophylactic anti-fungal treatment was added to the circuit.

Nakajima *et al.* investigated EVLP a platform to treat infected donor lungs with antibiotic therapy before lung transplantation[103]. Human donor lungs that were rejected for transplantation because of clinical concern regarding infection were randomly assigned to two groups. In the antibiotic group (n=8) lungs underwent

EVLP for 12 h with high-dose antibiotics (ciprofloxacin, azithromycin, vancomycin, and meropenem). In the control group (n=7), lungs underwent EVLP for 12h without antibiotics. A quantitative decrease in bacterial counts in BAL was found in all antibiotic-treated cases but in only two control cases. Perfusate endotoxin levels at 12 h were significantly lower in the antibiotic group compared with the control group. EVLP with broad-spectrum antibiotic therapy significantly improved pulmonary oxygenation and compliance and reduced pulmonary vascular resistance. Perfusate endotoxin levels at 12 h were strongly correlated with levels of perfusate TNF-α, IL-1β and macrophage inflammatory proteins (MIP) -1α and -1β at 12 h. Nakajima *et al.* concluded that EVLP treatment of infected donor lungs with broad-spectrum antibiotics significantly reduces BAL bacterial counts and endotoxin levels and improved donor lung function.

Cypel *et al*. investigated the potential to use gene therapy during EVLP, using adenoviral vector encoding human IL-10, an anti-inflammatory cytokine. After 12 hours on EVLP, the intra-airway adenoviral vector encoding IL-10 treated lungs showed significant improvement in Pa02/Fi02 and pulmonary vascular resistance when compared to a control group. There was also a shift from pro-inflammatory cytokine expression[82].

The use of bone-marrow-derived multipotent mesenchymal stem cells (MSCs) as treatment during EVLP has also been investigated. Lee *et al.* tested the therapeutic capacity of human MSCs to restore alveolar epithelial fluid transport and lung fluid balance from ALI in an EVLP human lung preparation injured by E. coli endotoxin[104]. Intra-bronchial instillation of endotoxin into the distal airspaces resulted in pulmonary oedema with the loss of alveolar epithelial fluid transport measured as alveolar fluid clearance. Treatment with allogeneic human MSCs or its conditioned medium given one hour following endotoxin-induced lung injury reduced extravascular lung water, improved lung endothelial barrier permeability and restored alveolar fluid clearance. They showed that instillation of bone-marrow derived multipotent mesenchymal stem cells resulted in decreased endothelial permeability. Mayes *et al.* investigated the role of the Human Amnion Epithelial Cell (hAECs) secretome a new potential treatment preventing neutrophil recruitment and endothelial expression during reperfusion[105]. This may decrease the risk of developing PGD after transplantation if treatment is prophylactically given during

EVLP. The hAECs were isolated from the healthy placentas from term women undergoing elective caesarean section. Pre-term placentas under the same ethical approval were collected from women undergoing planned caesarean section for reasons such as multiparity and placenta previa. Mayes *et al.* demonstrated that hAECs can inhibit the pro-inflammatory cytokine response and that these changes result in improved lung function. They demonstrated they hAECs may be able to inhibit neutrophil recruitment and reduce endothelial activation and showed that levels of TNF-α, lL-1 and IL-6 were reduced. A reduction in ICAM-1 and VCAM was also seen. The secreted products of hAECs reduce endothelial activation and limit leukocyte-endothelial interactions and may provide a therapeutic option during EVLP that protects against PGD.

Moreover, the utilisation of EVLP as a platform to provide different medications was examined. Nakajima *et al.* added nitroglycerin and dibutyryl cyclic adenosine monophosphate to Steen solution during EVLP of lungs subjected to 4 hours of warm ischaemia[106]. Subsequent to single LTx, EVLP lungs showed improved function, lower histological signs of acute lung injury as well as better microvascular patency in comparison to standard preservation lungs. Mulloy *et al.,* on the other hand, in a model of 1 hour of warm ischaemia in pigs added a selective adenosine 2A agonist to the perfusate[107]. Adenosine A_{2B} receptor antagonists have been shown in animal studies to improve physiological lung parameters when administered during EVLP[108-111]. Hijiya *et al.* in a canine model of EVLP reported that high-dose nebulised procaterol, a short-acting β2-adrenergic receptor agonist, during EVLP ameliorated lung graft dysfunction at the early post-transplantation period without severe adverse effects[112, 113].

Key processes of reperfusion injury include the formation of reactive oxygen species (ROS)/nitrogen species (RNS) and the activation of poly (adenosine diphosphate-ribose) polymerase (PARP). Some groups have explored whether rat lungs could be reconditioned during EVLP using the PARP inhibitor 3-aminobenzamide (3-AB) and demonstrated attenuation of lung weight gain and perivascular oedema with the inhibitor treatment[114].

Martens *et al.* investigated the effects of argon and xenon gases delivered during EVLP on porcine donor lungs and did not find any improvements[115, 116]. Haam *et*

al. did however show that hydrogen gas inhalation during EVLP improved donation after cardiac death lung function via reduction of inflammation and apoptosis, and this effect persisted after lung transplantation[117, 118].

Several animal studies have investigated the effects of surfactant as a lung reconditioning agent[119, 120]. Nakajima *et al.* showed that lung lavage, followed by surfactant replacement during EVLP, reduced inflammatory mediators and prevented hydrolysis of phosphatidylcholine, which contributed to the superior post-transplant function in donor lungs with aspiration injury[121].

Today, even the most aggressive lung transplant programs use at most 40% of offered donor lungs for transplantation. The remainder are either felt to be or are actually too injured to be safely utilised for transplantation. With the development of prolonged EVLP, there now exists great potential for: 1) the evaluation of questionable donor lungs and 2) the individualised repair of injured human lungs during the lung preservation phase. Successful development of this paradigm would greatly increase lung transplant volumes and reduce waitlist times and mortality. The potential for adding medications, gene therapies and immune modulating therapies to the EVLP circuit is vast. EVLP as a platform for drug delivery is an exciting concept which will be explored in future studies internationally.

Aims, Objectives, and Hypotheses

Aim

The aim of this MD thesis is to develop and evaluate novel methods of ex vivo lung perfusion as an approach for assessing the function of extended-criteria organs, and as a strategy for delivering and evaluating novel therapeutic interventions to improve donor organ quality.

Hypotheses

Hypothesis 1: Inflammatory and tissue injury markers of ex-vivo lung perfusion and lung reconditioning. Using transcriptomics to generate biomarkers that identify lungs suitable for transplantation following ex-vivo perfusion

I hypothesise that transcriptomic studies can be used as a method of identifying potential biomarkers, pro-inflammatory proteins detectable in perfusate and be able to predict the likelihood of human donor lungs undergoing successful ex-vivo reconditioning.

Hypothesis 2: Differences in the inflammatory profiles between donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD)

I hypothesise that there will be differences in the profiles of inflammatory and tissue injury markers between donor lungs undergoing EVLP following donation after brain-death (DBD) and donation after circulatory death (DCD). I hypothesise that DBD donor lungs will express higher levels of acute inflammation and tissue injury than lungs from DCD donors due to the insult of brainstem death and its associated haemodynamic instability, vascular endothelial injury and an acute inflammatory response in the lungs.

Hypothesis 3: The use of EVLP as a therapeutic platform for the reconditioning of human donor lungs.

I hypothesise that EVLP can be used as a therapeutic platform to assess the effect of sildenafil on human donor lungs that have been turned down for immediate transplantation. I hypothesise that sildenafil, a well-studied vasodilator will improve pulmonary physiology and lung function when administered during EVLP.

Objectives

In order to investigate my hypotheses, I will:

1) Analyse a panel of inflammatory markers in perfusate that have been identified through lung transcriptomics from clinical EVLP donor lungs and

investigate if a biomarker signal can be developed that distinguishes those donor lungs amenable to reconditioning by EVLP. Examine laboratory microbiology culture results for identification and quantification of bacterial and fungal species to evaluate how the microbial load in BAL samples is affected by normothermic lung perfusion with a perfusate containing high-dose antibiotics.

2) Examine a panel of inflammatory and tissue viability markers in perfusate, BAL, and lung tissue samples taken from clinical EVLP donor lungs and investigate if there are differences in the profile of markers between donor lungs following donation after brain death compared with those after circulatory death.
3) Develop a research EVLP model to be used as a therapeutic platform for reconditioning donor lungs turned down for immediate transplantation.
4) Analyse the effect of sildenafil, given during EVLP, on the pulmonary physiology, cytokine profiling and makers of endothelial disruption.

CHAPTER 2

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Materials and Methods

Chapter 2. Materials and Methods

Introduction

In this chapter, a detailed overview of the methodological techniques used during this MD project is presented. Each results chapter contains its own individual materials and methods section, allowing for a more detailed and specific account.

Materials & Methods

Porcine procurement

As part of initial training in EVLP, I utilised 2 key streams of animal work in order to gain knowledge and expertise in procurement of the lungs, transportation, dissection and attachment of the lungs to the EVLP machine. I gained training in the procurement of porcine lungs from animals sacrificed under schedule 1 practices meant for the food chain by attending *Thompson Wholesale Meat Ltd Abattoir* in Bishop Auckland, United Kingdom. Prior to my arrival to the research group, there was already an established working relationship between the management at this commercial abattoir and experienced Cardiothoracic Surgeon; Mr. Tanveer Butt. Mr. Butt had procured porcine lungs for the purposes of surgical training and "wet-lab" surgical training days held at the Freeman Hospital, Newcastle. The porcine model offers very appropriate size comparisons to humans. Because of this, comparable tidal volumes, PEEP, and perfusion times can be used for the EVLP. As a result, information obtained in this large animal model of can be rapidly and directly transferred to settings for human studies. Training at the abattoir alongside Mr. Butt involved dissection of a porcine heart-lung bloc with identification of the main blood vessels, the pulmonary artery (PA) and aorta. I learnt the steps involved in efficiently dissecting the lungs from a porcine heart – lung bloc and then carefully suturing a cannula into the pulmonary artery for antegrade and retrograde flushing of the lungs. I quickly became independent in the procurement of porcine lungs and confident in the dissection of the pulmonary vessels which allowed me to use these animal organs as a valuable tool for training in the set-up and running of the EVLP circuit. Additionally, I have carried out approximately 8 ovine EVLPs in conjunction with the Edinburgh EVLP team who procure their animals from the *Roslin Institute, University of Edinburgh* for the ENLIGHTEN project.

All porcine procurement was carried out at *Thompson Wholesale Meat Ltd Abattoir,* Marshall Green Abattoir, Bishop Auckland DL14 0AQ. Arrival was typically ~11am in order to coincide with the culling of pigs. The porcine procurement kit including all necessary instruments can be found in **Table 2.1**. Both male and female pigs weighing

between 60 and 80 kilograms were euthanized by abattoir staff before exsanguination and evisceration; providing a DCD model. Lungs were removed by staff as a heart-lung-liver block and handed over for further dissection. Dissection began with the removal of unnecessary tissue such as the liver, oesophagus, descending aorta, heart. The heart was dissected along the atrioventricular border and then along the interatrial septum, removing ventricles and leaving atrial walls. PA and aorta were separated, and aorta was dissected off but retained. The heart was then removed leaving a left atrial cuff and a single common pulmonary artery. Any visible thrombus was physically removed. After initial dissection, 2 litres of 0.9% Sodium Chloride per pair of lungs, pre heparinised with 2,500 units/litre of unfractionated heparin was given as an antegrade and retrograde flush with an aim to remove all evidence of thrombus and have the flushing liquid “run clear” indicating removal of donor blood from the lungs. The initial litre bag of fluid at room temperature and second litre at 4°C. Lungs were intubated with an 8.0 mm endotracheal tube (ET), and ventilated during flushing of the first two litres of saline using a portable Ambu bag hand ventilator. Lungs were not ventilated during the final litre to avoid mechanical damage associated with ventilation of cold lung tissue. Lungs were transported partially inflated, within an organ transport bag on ice back to our lab in the Transplant Regenerative Medicine Facility, NHS-BT Donor Centre, Holland Drive, Newcastle, for perfusion. Exclusion criteria during procurement were based on visual inspection of lungs. Any lungs with contusion, lacerations, signs of consolidation, or oedema (at any point, including during flush) were rejected.

Porcine Procurement Kit – replenished after each procurement
• Protective, aseptic clothing: surgical scrubs, gloves, gowns, shoe covers
• 1 X research retrieval box filled with ice.
• 4 X litre bags of cold 0.9% Sodium Chloride
• 2,500 units of unfractionated heparin/litre of Saline prepared prior to arrival
• Surgical instrument kit: **See Figure 2.3** (2 of each surgical instrument shown)
• 2 X endo-tracheal tube (size 8.0)
• Ambu bag for ventilation
• Blood giving set
• Pulmonary artery flushing catheter
• 2 X 10 ml and 50 ml syringes
• Silk ties 1.0 or 2.0

Table 2.1 Porcine procurement kit

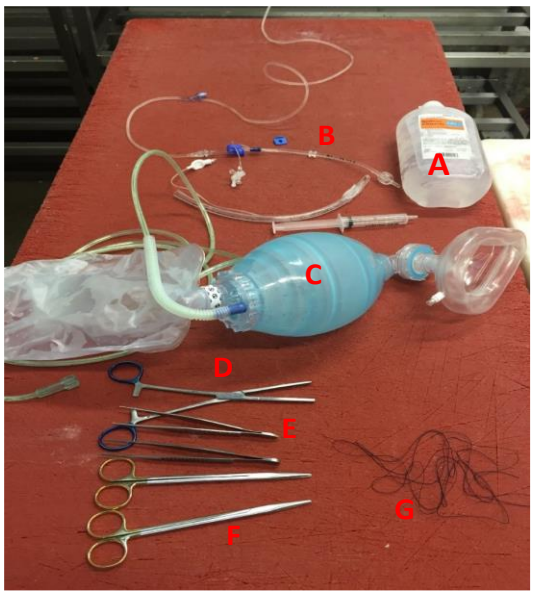

Figure 2.1 Work station for porcine lung procurement

Equipment used during porcine procurement. (A) 1 Litre bag of cold saline heparinised with 2,500 units; (B) Pulmonary artery flushing catheter; (C) Bag-mask ventilator (D) Needle holders (E) Forceps (F) Scissors (G) 2.0 Silk ties.

Figure 2.2 Procurement instruments

Procurement instruments: A - Mayo scissors, straight, (Precision surgical, 51-1830). B - Metz scissors (BOSS, 50-1751). C - Probe 180MM (Precision surgical, 13-9110). D - Debakey vascular tissue forceps (Precision surgical, 65-2241). E – Tissue forceps, 2x3 teeth (BOSS, 10-4125). F - Crile forceps, (BOSS, 14-1266). G – Portable Ambu bag.

There have been many learning points from repeated procurements at the abattoir. There are often lung contusions, lacerations and damage to the pleura visible on initial inspection of the lungs. This is most likely due to the method in which the organs are removed from the pig and it can be highly variable depending on the staff member responsible. Having a more experienced abattoir technician has been beneficial. I have found that explaining to the abattoir workers what my research involves and demonstrating to them the technique of flushing the lungs has helped build a good rapport. I have then been able to emphasise the importance of avoiding lacerations through the slow and careful removal of the organs from the pig. The lungs often show gross, frank pulmonary oedema from the outset and it is hypothesised that there is shear stress from the removal of the lungs from the pig thoracic cage which may lead to damage to the vasculature and loss of vascular integrity.

Another problem that has been highlighted during the porcine procurements is the variability of the warm ischaemic times. There are also steps in the abattoir's culling protocol that involves immersing the pigs in a large tank of water at 80°F. This allows for any debris to be removed. The pigs are then scorched with flames to singe the remaining hair from the carcass. This likely does not impact visceral or thoracic organ temperature dramatically due to skin and fat insulation; however, it undoubtedly results in an exaggerated warm ischaemic time and if any breathing were to occur may result in damage to airways and so has been taken into consideration whilst explaining the severe pulmonary oedema seen in some cases. Despite this, the porcine procurement offers a vital training opportunity. The procurement at the *Roslin Institute* allows for a more standardised protocol. It is the same individual who culls the animals on every occasion and more time and care is put into retrieving the lungs as the time allows. Experience shows that there has been less oedema seen from the outset in the ovine lungs versus the porcine lungs and ovine lungs have shown greater stability over the duration of the EVLP. Although there has not been a direct experimental comparison. I postulate it is a direct result of differences in the culling methods and warm ischaemic time.

Perfusion Laboratory set up

In the initial 6 months of my research project I worked closely with the clinical perfusionists at The Freeman Hospital who have a wealth of experience in clinical EVLP following their integral role in the DEVELOP-UK study. From discussions and shadowing exercises I developed an appropriate list of permanent equipment, laboratory consumables, additive medications and required gases for establishing a new perfusion laboratory space for research only EVLP. I procured all necessary consumables and set up a functional perfusion lab with the necessary stock, equipment and consumables based at NHS Blood Donor Centre, Holland Drive, Newcastle NE2 4NQ. I received training in the priming of an EVLP circuit, setting up gas cylinders and attended a gas safety training course.

I have produced the relevant risk assessments for both the animal EVLP model and the human research EVLPs. This involved developing BioCoSHH forms to outline the risks associated with the activity being carried out in the lab and a description of the action taken to reduce these risks and protocols in place. Risk Assessments were also completed for the oxygen, mixed gases and medical air.

Developing an EVLP model

When deciding on which EVLP model to use for prospective studies there were several factors that had to be considered. The Vivoline® LS1 (Vivoline Medical, Lund, Sweden) EVLP machine had been used during the DEVELOP-UK trial and there was local knowledge of its set-up, design and troubleshooting of problems. The Vivoline EVLP machine was a commercially available kit with one major downfall, the cost of its consumables, in the context of its use within a research project. The consumable in which the lung is reconditioned is costed at £6963 for a non-sterile Vivoline disposable lung set. Following on from DEVELOP-UK, it has been well recognised that clinical EVLP is an expensive procedure due to the accumulative costs of staff, equipment, consumables, theatre usage and drug used during EVLP. The cost of the EVLP procedure was found to be £42,633 (mean). When considering which kit to use for training and research purposes we looked to reduce these costings.

The Vivoline LS1 is automated and has one phase for lung reconditioning and one for evaluation of lung function. During reconditioning, the oxygenator is supplied with a gas mixture of nitrogen 74%, oxygen 21% and carbon dioxide 5%. During the evaluation phase, the oxygen supply is disconnected, and the oxygenator is used to

deoxygenate the perfusate in the EVLP system with a gas mixture of 93% nitrogen and 7% carbon dioxide.

In order to gain training and experience with setting up the EVLP machine, priming the circuit, attaching the lungs and ensuring the circuit was set-up correctly I used the existing research Vivoline machine at Freeman Hospital perfusion lab. In order to reduce the costs of purchasing new lung set inserts, we decontaminated each set after each porcine EVLP and reused this equipment. The machine and all consumables was labelled clearly for “Research Use Only”.

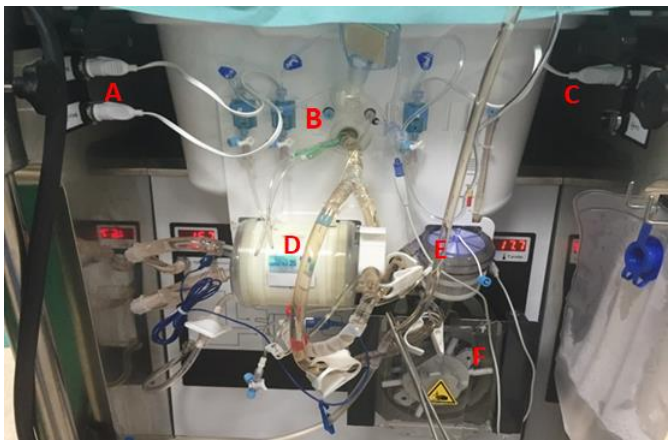

Figure 2.3 Posterior View of Vivoline Machine

Posterior view of Vivoline machine set-up during EVLP. (A) Pulmonary artery pressure transducers (B) Entry port for bronchoscope (C) Temperature transducer line (D) Oxygenator (E) Leucocyte filter (F) Roller pump

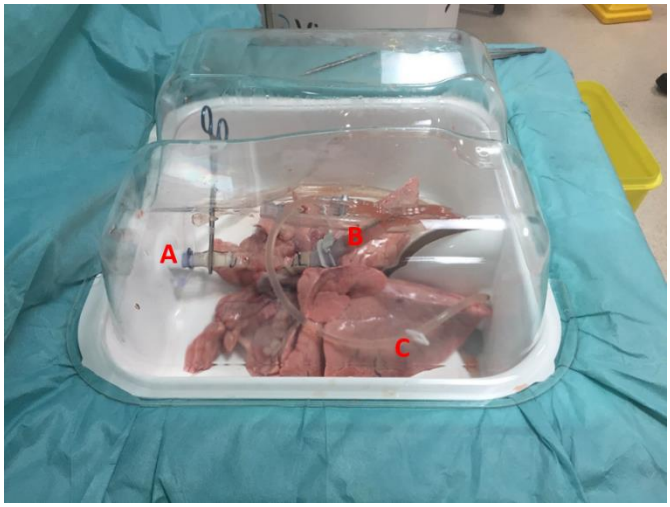

Figure 2.4 View of Vivoline Machine from above

View of Vivoline machine from above. Lungs are placed in a lung basin and kept covered with a clear plastic hood in order to preserve heat and prevent drying out of the lungs. (A) Endo-tracheal tube clamped prior to commencing ventilation (B) Pulmonary artery cannula (C) Shunt in closed position.

Due to cost implications it became clear that a more economical model would need to be sought. Liaising with the Abdominal Perfusion team at Freeman Hospital links were made with Medtronic™, one of the largest multi-national medical device companies in the world with a portfolio spanning clinical services in neuroscience, vascular catheters and cardio-pulmonary bypass equipment. A pre-existing design of an abdominal perfusion circuit was modified and additional features added, such as

capacity for a leucocyte filter, **Appendix B**. The Medtronic kit, seen in **Figure 2.5**, includes all the necessary component parts of the EVLP circuit and is also set up with a computer console to monitor parameters such as pulmonary artery pressure, flow rate and temperature during the EVLP. The Medtronic Kit comprises a reservoir, oxygenator, centrifugal pump with 3/8 tubing connecting the component parts. The Medtronic software allows for in-line pressure monitoring via transducers placed within the pulmonary artery. The software ensures continuous monitoring of flow (l/min), RPM of the centrifugal pump and PAP (mmHg). The Medtronic kit is costed at £680 per kit and so is 10% of the cost of the Vivoline LS1 lung insert. The costings breakdown and total cost for Medtronic EVLP for all human donor lungs included in the Novel Therapeutics study can be seen in **Appendix C.**

Figure 2.5 Medtronic EVLP circuit

Medtronic EVLP system. (A) Touch-screen Monitor (B) Console (C) Adult reservoir (D) Leucocyte filter (E) Centrifugal pump head (F) Flow meter (G) 3/8"Tubing. In addition to these items supplied by Medtronic, the following items were used: (H) Ventilator (I) Vivoline LS1 lung basin and dome. (J) Medtronic Kit as packaged.

Standard operating procedures for EVLP

In conjunction with initial training using porcine lungs and developing a cost effective and reproducible EVLP set-up, I developed standard operating procedures for all the individual elements of EVLP. I designed a logistics algorithm which was presented to and approved by the Northern Specialist Nurse in Organ Donation (SNOD) team and the National Organ Retrieval Service (NORS), **Appendix D**. This logistics algorithm laid out the pathway in which lungs which had been turned down for transplantation would be able to be used for research purposes.

Potential organ donors were to be identified and consented by SNODs for research according to the existing policies of the NHS-Blood and Transplant (NHS-BT). Potential organ donors are referred to SNODs by local critical care units. Once the suitability for organ donation is confirmed, the family is approached and consent for clinical and research use is obtained. Once consent is obtained and donor assessment performed, the patient is registered via the NHS-BT Duty Office prior to offering organs. The Duty Office and/or SNOD will ensure that all potential donor organs are offered to the recipient centre point of contact (RcPoC) at national transplant centres; subject to absolute contraindications. Organs may be placed into this research programme via 2 mechanisms:

- If an organ is retrieved and deemed unsuitable for clinical use upon back-bench inspection and the family has consented for generic research; it will be referred to the NHS-BT duty office for placement in a nationally registered study with appropriate REC approval.

- If an organ is not accepted by any centres for clinical use, study specific consent has been obtained, and the donation occurs in a Human Tissue Authority (HTA) licensed facility; the organ may be procured explicitly for use in this study.

Once tissue has been accepted into the study, suitable licensed transport will be arranged and tissues transported to the designated facility. All tissue will be appropriately disposed of according to HTA guidelines within 7 days or sent on to a designated location for long term storage.

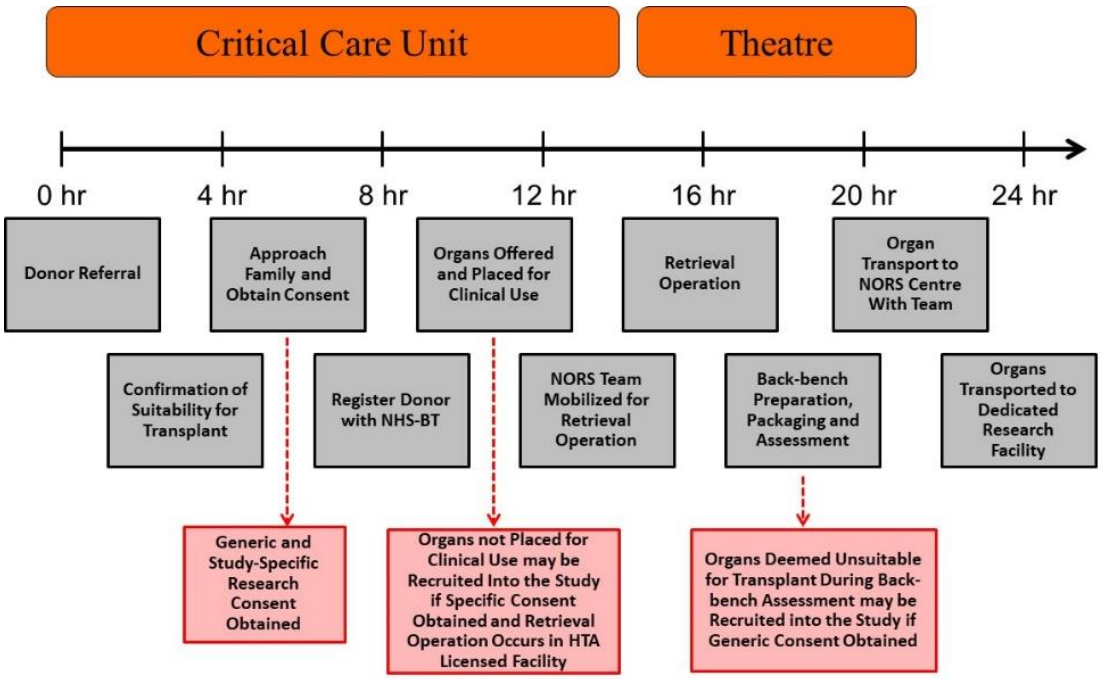

Figure 2.6 Schematic Timeline Summarising Key Steps for Recruitment of Organs to this Study

Adapted from the BTRU Study protocol, version 1.2, 30th January 2017. An algorithm and timeline showing the process from when a potential donor has been highlighted.

INCLUSION CRITERIA	EXCLUSION CRITERIA
• Patient identified as a potential organ donor by the NHS-BT • Age between 18-85 years • Must fall into one of the following groups: defined brain death or individuals who have had a withdrawal of care and a minimum 5-minute observation period to confirm cardiac death. • Only patients with appropriate consent reflecting their own and their families' wishes will be considered. • To be eligible for recruitment into the study, the tissue in question will have been declined for clinical use by all transplant centres in the UK.	• Under the age of 18 or above age 85 • HIV, Hepatitis B, Hepatitis C. Tuberculosis (TB), Clostridium difficile (CD) • Methicillin resistant staphylococcus aureus (MRSA).

Table 2.2 Study Inclusion/Exclusion criteria

Retrieval Procedure

The standard clinical lung procurement procedure was followed for donor lungs to be used for EVLP in the study. The Cardiothoracic Team would adopt usual practice when flushing the lungs on retrieval. The organs are anterogradely flushed with supplemented (3.6% THAM 3.3 ml, 0.6 ml CaCl +/- 2.5 ml Prostacyclin / litre) Perfadex®, the first one litre at room temperature, the rest at 4 °C. A minimum volume of 60 ml/kg will be given. After the anterograde dose, 200ml will be given down each pulmonary vein as a final retrograde flush.

An adequate portion of main PA, left atrial cuff, and at least 4 cm of trachea will be taken by the retrieval surgeon. A segment of aorta will be required to extend a deficient main PA (divided in close proximity to the bifurcation) to allow for successful cannulation and bilateral perfusion. The lungs will be transported on ice, in the standard way at retrieval in a “human tissue” ice box and be transported in the organ courier. EVLPs were carried out in the Transplant Regenerative Medicine Laboratory on the 3rd level of the NHS-BT building: Newcastle Blood Donor Centre, Holland Drive, NE2 4NQ.

Ex-vivo lung perfusion protocols

This thesis contains data from three separate studies investigating the use of EVLP to recondition rejected donor lungs in clinical lung transplantation. The retrospective studies that will be described in Chapters 3 and 4 were from the national multicentre study DEVELOP-UK study[98]. Using the Vivoline LS1 machine, DEVELOP-UK started off with a Hybrid protocol, so called because of its use of mixed features from the established Toronto and Lund protocols. After a pause of the DEVELOP-UK study and suspicion of protocol related poor perfusion, concerns regarding high requirements for post-operative ECMO and transplantation outcomes, the assessment was changed to the Lund protocol for which the LS1 machine was originally intended. The prospective novel therapeutics study that will be reported in Chapter 5, utilised a bespoke Medtronic EVLP circuit and a modified Toronto perfusion protocol. Below follows an outline of the three different perfusion protocols that will be referred to in this thesis, **Table 2.3.**

	HYBRID	LUND	TORONTO
STUDY GROUP	The DEVELOP-UK study (2012 - 2013)	The DEVELOP-UK study (2013 - 2014)	Prospective Novel therapeutics study (2017-2018)
PERFUSION			
Target flow	40-60% of cardiac output	100% of cardiac output (70 ml/kg/min)	40-60% of cardiac output
Pulmonary arterial pressure	<20 mmHg	<20 mmHg	<15 mmHg
Left atrial pressure	0 mm Hg (Open LA)	0 mm Hg (Open LA)	3-5 mm Hg
Pump	Roller	Roller	Centrifugal
Circuit	Vivoline LS1	Vivoline LS1	In-house Medtronic
Perfusate	2L Steen Solution™	2L Steen Solution™ with red-cell concentrates (Haematocrit 10-15%)	2.5L Steen Solution™
VENTILATION			
Mode	Volume controlled	Volume controlled	Volume controlled
Tidal volume	6-8 ml/kg	6-8 ml/kg	7 ml/kg
Frequency	10-15 bpm	10-15 bpm	7 bpm
PEEP	5 cm H_2O	5 cm H_2O	5 cm H_2O
FiO2	50 %	50 %	21 %
TEMPERATURE			
Start of ventilation	32°C	32°C	32°C
Start of perfusion	15°C	15°C	15°C
Start of evaluation	37°C	37°C	37°C

Table 2.3 Ex-vivo lung perfusion protocols used over the course of this MD project

A detailed, step by step, description of the EVLP set-up for DEVELOP-UK can be found in **Appendix E**. The procedure was performed using the Vivoline LS1 system (Vivoline Medical AB, Lund, Sweden). The circuit was primed with 2 litres of STEEN Solution with or without donor blood type specific leukocyte depleted packed red blood cells, depending on if following a cellular or acellular protocol. A target haematocrit of 10-15% was achieved. 10,000 iU heparin was added and the perfusate was buffered to a physiological level of pH of 7.35 – 7.45 with trometamol (THAM, Braun or Addex-THAM). Finally, 500 mg meropenem, 10 mg amphotericin B and 500 mg methylprednisolone were added to the perfusate. A blend of oxygen, nitrogen and carbon dioxide was supplied to the membrane oxygenator and the flow was adjusted to achieve a physiological venous blood gas. A visual inspection was carried out and recorded, to assess for areas of atelectasis, contusion, lacerations, areas of consolidation and tracheal secretions.

After a period of cold storage, the PA cannula (XVIVO Perfusion AB, Gothenburg, Sweden) was connected to the perfusion circuit with careful de-airing through the open bypass shunt. The left atrium was left open and visualised to ensure a smooth flow of perfusate. The LA temperature probe and sampling line was secured in place equal distance from the 4 pulmonary veins. Reperfusion was initiated at a low flow rate of 0.5 L/minute. The lungs were slowly re-warmed, maintaining a 8°C difference between lung temperature and perfusate temperature. The flow rate was increased whilst ensuring that PA pressure was initially maintained at 15mmHg and then if stable, at 20 mmHg. At 25 °C the bypass shunt was closed, diverting all of the perfusate through the lung vasculature. When the temperature of the lungs reached 32 °C, protective mechanical ventilation was initiated maintaining peak airway pressures of less than 20 mmHg. Ventilation was gradually increased as the lungs continued to warm, with full ventilation achieved at 37 °C. During this time, if there were areas of persistent atelectasis, recruitment manoeuvres were performed through transient increases in PEEP. When a steady state was achieved, with stable perfusate flow and ventilator settings, blood gases and haemodynamic parameters were registered. At this point the oxygenator was disconnected from the circuit, so that the perfusate was representative of mixed venous blood and the functionality of the lungs could be assessed via the oxygenation of the blood. An arterial and venous blood gas was run on a fraction of inspired oxygen (FiO2) of 50%, 100% and 21%. **Table 2.4** demonstrates the criteria for lungs to be deemed acceptable for

transplantation at the end of EVLP. If lungs were suitable then they were cooled, oxygen was reconnected, ventilation was stopped and the trachea was clamped at half inspiration.

The prospective novel therapeutics study of human lungs took place at the designated research area at the NHS Blood Donor Centre, Holland Drive, Newcastle, NE2 4NQ. A Toronto method was used involving an acellular perfusate, 40% of full cardiac output (70 ml/kg IBW/minute) and closed left atrium.

A modified, in-house assembled Medtronic circuit (bespoke design, M444426B, Appendix B) was used, made up of: Vivoline LS1 lung chamber, plastic hood, centrifugal pump, an adult oxygenator, a LeukoGuard leukocyte filter (Pall Corporation, Port Washington, NY), a HICO heater-cooler unit (HICO, Germany), Medtronic pressure sensors, and temperature probes.

Parameters to be met at EVLP Evaluation Stage
• PAP <20mmHg, whilst achieving at least a target flow of 40-60% of a calculated donor flow index.
• Oxygen capacity shown by delta PO2 of >40kPa (perfusate LA PO2 – perfusate PA PO2) / FiO2
• Selective PV gas > 30 kPa on 100% FiO2 and 5 cm H2O PEEP
• Stable or improving lung compliance and stable or falling lung resistance
• No pulmonary oedema build-up in the ET tube
• Satisfactory assessment on inspection and palpation

Table 2.4 Criteria for Successful EVLP Assessment

Sample collection and processing

This section contains the protocols for sample collection and processing of donor lung samples taken from three lung compartments: vascular compartment (perfusate fluid), airway compartment (BAL fluid), and tissue compartment (lung biopsy).

Perfusate

Samples of perfusate solution were collected longitudinally during the EVLP process. 5 ml were collected from the perfusate sampling port at the following time-points:

<u>All studies</u>

Perfusate 0	Taken from the primed EVLP circuit before the donor lung perfusion is started
Perfusate 1	Taken 15 minutes after perfusion is started
Perfusate 2	Taken 30 minutes after perfusion is started
Perfusate 3- maximum Perfusate 8	Taken every 30 minutes during perfusion
Perfusate X	Taken at the end of the perfusion immediately before the perfusion is stopped

The perfusate samples were centrifuged to remove cellular debris and aliquoted equally into 5 x 1 ml tubes before being frozen initially at -20^{o}C and then transferred to a -80^{o}C for longer term storage and subsequent laboratory analysis.

Bronchoalveolar lavage fluid

As part of the study protocol, under flexible bronchoscopic guidance a standardised BAL using 120 ml of sterile normal saline (0.9% sodium chloride solution) was performed from either the left or right lower lobe of the donor lung on two occasions (BAL1 and BAL2). BAL2 was performed from the same lobe as BAL1, but in a different segment of that lobe. The timing of each BAL is detailed below:

BAL 1: At the beginning of the EVLP process after perfusion has commenced and the lung temperature has reached at least 30^{o}C, but before ventilation of the lung is initiated.

BAL 2: At the end of EVLP process once the final assessment is complete but before ventilation is discontinued.

The BAL was filtered through gauze to remove excess mucus and then centrifuged to separate the cellular component from the acellular supernatant. The acellular supernatant was divided into 1 ml aliquots in storage tubes. The storage tubes were then frozen initially at -20ºC and then transferred to a -80ºC for longer term storage and subsequent laboratory analysis.

Lung tissue biopsies

Small biopsies (approximately 3x3x1 cm) of lung tissue were taken using a Covidien Duet (absorbable buttressed) endo-GIA stapler from either the right middle lobe or lingula

For DEVELOP-UK, at two time points:

Biopsy Pre: Taken prior to commencement of the EVLP process

Biopsy Post: Taken at the end of the EVLP process once perfusion has stopped from the same location as Biopsy 1.

For novel therapeutic study, at four time points:

Biopsy 1: Taken prior to commencement of the EVLP process

Biopsy 2: 30 minutes' post drug dosing

Biopsy 3: 60 minutes' post drug dosing

Biopsy 4: Taken at the end of the EVLP process once perfusion has stopped from the same location as Biopsy 1.

Biopsies were placed on sterile gauze dampened with 0.9% sodium chloride in a sample pot and the pot stored on ice until processing. From each of these biopsies, 2 pieces of tissue, approximately 5-10 mm in diameter were snap frozen using dry ice and isopentane slurry, for subsequent mechanistic studies, stored at -80°C. The remaining tissue was fixed in formalin, paraffin embedded and sections cut for routine histological evaluation. All fixing was carried out by the Cellular Pathology Research Laboratory, Royal Victoria Infirmary, Newcastle upon Tyne. Tissue blocks were available for subsequent immune-localisation studies using immunohistochemistry.

For protein analysis snap frozen lung tissue (100 mg/sample) was homogenized in 500 µL homogenization buffer (2 tablets complete™ mini protease inhibitor (Roche

Diagnostics, Basel, Switzerland) in 10 ml ready-to-use Radio-Immunoprecipitation Assay (RIPA) Buffer (Sigma-Aldrich, St Lois, MO, USA)) using a Minilys® ceramic bead tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Each homogenate was centrifuged twice at 13,000 g for 5 minutes, the supernatants decanted, and the protein concentration of each sample estimated using a Pierce™ BCA protein assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) before storage at -80^{o}C. 4 mg protein/ml diluted in 0.1% BSA in homogenization buffer was loaded onto protein assay plates according to manufacturers' instructions.

Sampling Protocol

Throughout the EVLP there are samples including perfusate, BAL and tissue biopsy taken for future analysis. Below in **Figure 2.7,** the time points at which these are taken in relation to the stage of EVLP is shown.

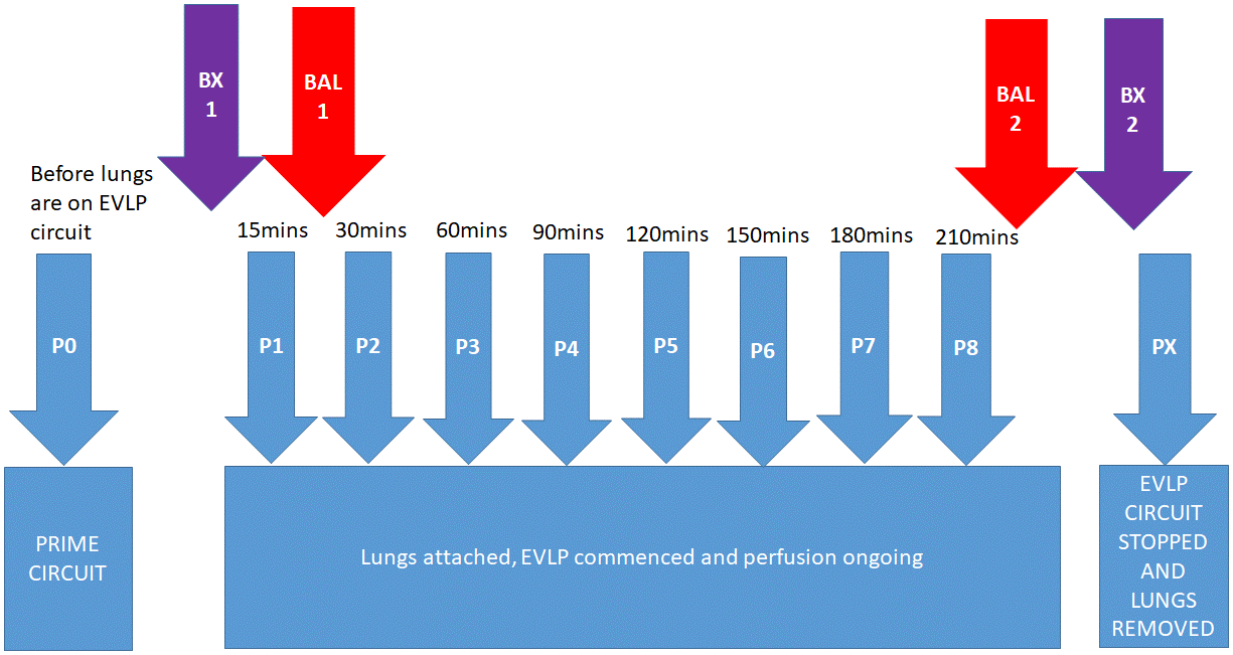

Figure 2.7 Flow chart for sampling protocol during EVLP.

Bronchoscopy will be performed on 2 occasions during a standard EVLP: 1ST at the beginning of EVLP, perfusing the lungs but before ventilation, lung temperature 30°C. 2ND at the end of EVLP but before ventilation stopped. The BAL samples will be taken from the RLL (right lower lobe) and/or LLL (left lower lobe), using up to 120 ml of 0.9% Saline per lavage. Lung biopsies could be taken from the RML or lingual at pre and post EVLP using a Covidien Gia Surgical Stapler.

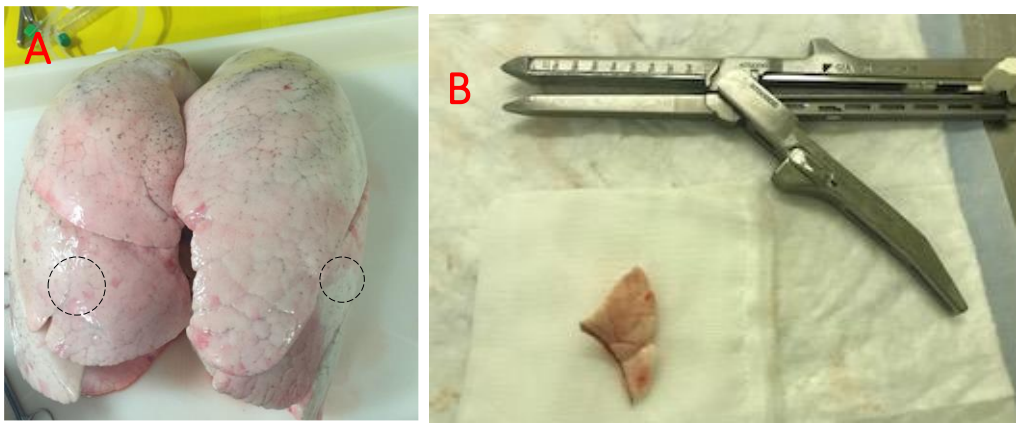

Figure 2.8 Pair of Lungs with markers of biopsy sites

(A) A pair of human lungs declined for transplantation prior to EVLP. Dotted circles indicate biopsy sites. Lung biopsies taken from the RML or lingual at pre and post EVLP using a Covidien Gia Surgical Stapler (B).

An alternative perfusate to Steen Solution

During all human EVLPs we used commercially available Steen Solution, a perfusate which is an extracellular solution with the addition of human albumin to maintain optimal colloid pressure and dextran 40 to protect the endothelium from complement and cell-mediated injury and to inhibit coagulation and platelet aggregation. A minimum of 2 litres of Steen is needed for each EVLP with the potential for additional 500 ml hourly exchanges. It is produced by XVIVO Perfusion Sweden at a cost of €876 per 500 ml bottle. With an overall cost per experiment of €3504 without exchanges. Due to this cost, an alternative solution was sought which could be used for animal work and training purposes. I liaised with the research team at Manchester University, James Fildes and William Critchley who advised us on the constituents and procedure for producing an alternative perfusate to the commercially available Steen Solution. The protocol for making this alternative STEEN can be found in **Appendix F.** The cost of alternative Steen solution is £200 per 2 litres and is therefore far more economical for porcine and training purposes.

Quantification of protein expressions in perfusate, bronchoalveolar lavage fluid and lung tissue lysates

Enzyme-linked Immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technology used to detect and quantify substances such as proteins and hormones. In this project I have consistently used commercially available already optimised sandwich

ELISA kits, most commonly DuoSet® ELISA development systems by R&D (Bio-techne, Minneapolis, MN). This type of ELISA assay is called a “sandwich” ELISA as the measured protein is bound between two primary antibodies – a capture antibody and a detection antibody, **Figure 2.9.** The basic technique of a sandwich ELISA consists of coating the wells of 96 well polystyrene plates with an antigen specific capture antibody that passively attaches to the plastic of the well. The sample is then added and the attached capture antibody used to immobilize any antigen that comes in contact with the surface of the well and its attached antibodies. After washing out excessive sample, separating bound from non-bound, the amount of antigen bound by the primary antibodies can be quantified by the addition and complex binding of an antigen specific detection antibody linked to an enzyme. This ability to wash away non-specifically bound materials and detect proteins specifically bound to the primary antibodies on the bottom of the well makes the ELISA a powerful tool for measuring specific proteins within a crude preparation, such as perfusate, BAL fluid or tissue lysates. Detection and quantification of proteins in the sample is accomplished by measuring the activity of the enzyme linked to the detection antibodies by incubating it with a substrate for 15-20 min to produce a light signal that can be measured with high sensitivity. The most commonly used enzyme label is horseradish peroxidase (HRP) which catalyses the conversion of tetramethylbenzidine (TMB) into a measurable coloured product. The final quantification of sample protein concentration is established by the creation of a standard curve, which is prepared by serial dilutions of a known quantity of the measured protein across a range of concentrations near the expected sample concentration. The protein content of the unknown perfusate, BAL or tissue lysate sample is determined by interpolation from the generated standard curve. **Appendix G** gives a detailed, step-by step description of the ELISA technique used in this thesis.

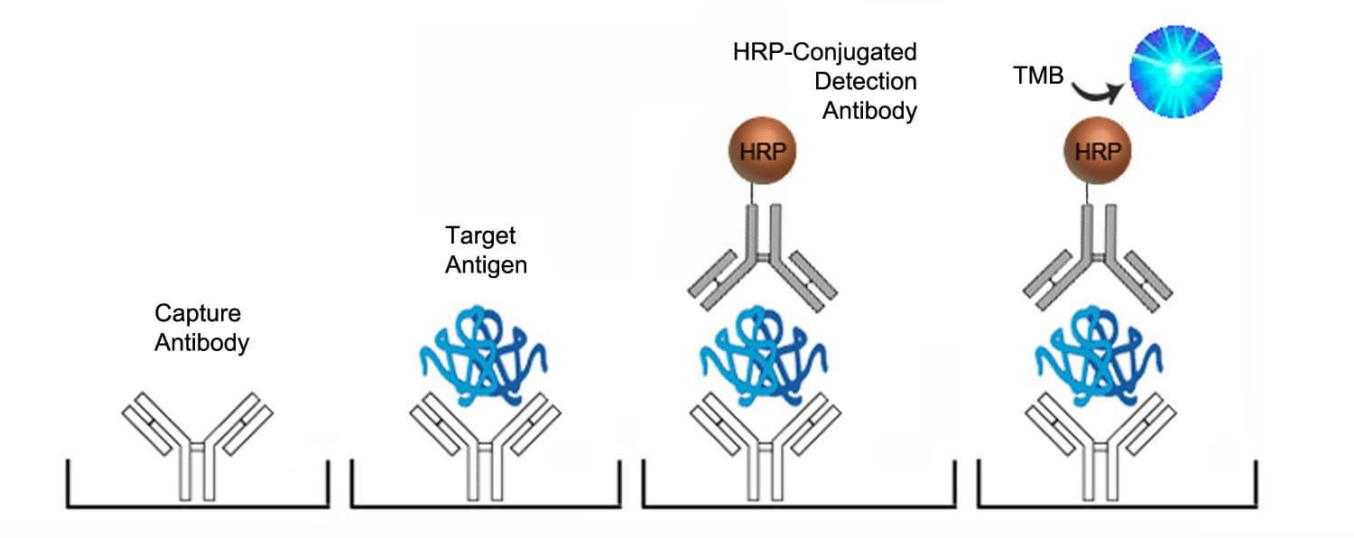

Figure 2.9 Diagram of a sandwich enzyme-linked immunosorbent assay (ELISA)

Diagram of a sandwich enzyme-linked immunosorbent assay (ELISA). In the assay, the protein of interest is immobilized by first attaching a capture antibody to the plate surface. Detection and quantification of the antigen is then accomplished by using an enzyme-conjugated primary antibody. Adapted from LS Bio LifeSpan Biosciences, Inc[122].

Meso scale discovery multiarray

A wide range of protein markers were analysed in perfusate, BAL, and tissue lysate samples with electrochemi-luminescent Meso Scale Discovery (MSD) Multi-Array® (Meso Scale Diagnostics, LLC, Rockville, MD). The assay was performed according to manufacturer's instructions with technical support from an experienced MSD technician. The MSD assay follows similar procedural steps as an ELISA. Briefly, multiarray plates pre-coated with capture antibodies for up to ten human proteins are incubated with standards and samples. After two hours of incubation and passive complex binding of protein to the specific capture antibodies fixed to the bottom of the well, a detection reagent containing electrochemi-luminescent labels conjugated to detection antibodies is added. Electricity applied to the plate electrodes by an MSD instrument leads to light emission by the "SULFO-TAG" labels Light intensity is then measured and compared to a standard curve to quantify protein levels in the sample, **Figure 2.10** [103].

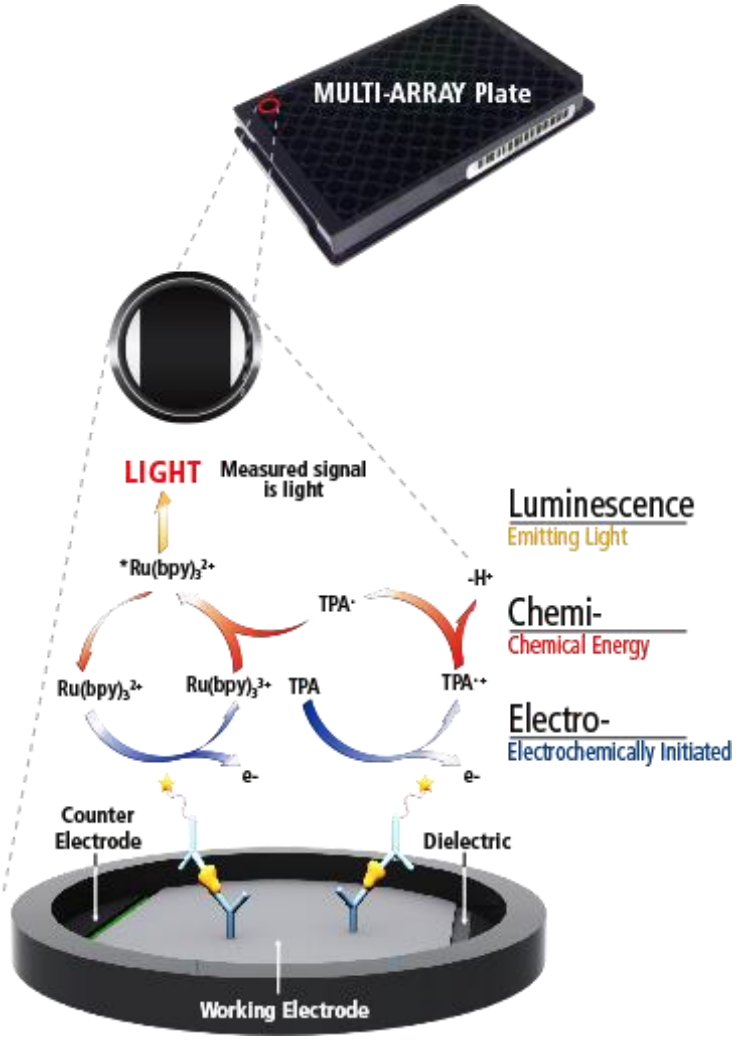

Figure 2.10 MSD assay

High binding carbon electrodes in the bottom of the MSD microplates allow for attachment of biological reagents. MSD assays use electrochemi-luminescent labels (SULFO-TAG) that are conjugated to detection antibodies allowing ultra-sensitive detection. Electricity applied to the plate stimulates light emission from the labels. Light intensity is then measured to quantify sample protein levels [103].

Immunohistochemistry staining of lung tissue

Antibodies used for immunohistochemistry were as follows: IHC primary Ab: anti-eNOS (ab5589, Abcam, Cambridge, MA) and Neutrophil Elastase (42 mg/L Clone NP57 DAKO, Glostrup, Denmark)

Tissue from biopsies taken pre, during and post-EVLP, see sample protocol and collection section. Tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. 5-µm sections were cut and mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, USA). Following de-waxing and citrate antigen unmasking, eNOS and Neutrophil Elastase (NE) were detected in parallel single-stain experiments.

NE antigen was retrieved using trypsin (BD, New Jersey, USA), incubated at 37.5oC for 20 minutes. Monoclonal mouse anti-human antibodies against NE (42mg/L Clone NP57 DAKO, Glostrup, Denmark), was used in 1:100 concentrations and incubated for 90 minutes at RT. A Dako EnVision+ System, HRP (DAKO, K4006, Glostrup, Denmark) was used as secondary antibody and protein visualisation.

eNOS antigen was retrieved by incubating in boiling citrate buffered to pH6, heated in a microwave on full power for 10 minutes. Rabbit polyclonal to eNOS was used in 1:50 concentrations incubated at room temperature for 1 hour and then overnight at 4°C, in the fridge for 18 hours. A Dako EnVision+ System, HRP (DAKO, Glostrup, Denmark) was used as secondary antibody and protein visualisation.

Proteins were visualized by avidin-biotin method with biotinylated secondary antibodies and subsequent DAB chromogen staining (Vector Labs, Burlingame, CA). Sections were counterstained with Mayer's haematoxylin, dehydrated, cover slipped, and digitally photographed. **Appendix H** provides a detailed, step by step description of the IHC protocols used in this thesis.

RNA Sequencing

The RNA sequencing results shown in Chapter 3: Novel Biomarkers of Ex-Vivo Lung Perfusion, were carried out by our BTRU Cambridge collaborators Dr Menna Clatworthy and Dr John Ferdinand. RNA sequencing (RNA-Seq) uses the capabilities of high-throughput sequencing methods to provide insight into the transcriptome of a cell. A typical RNA-Seq experiment consists of isolating RNA, converting it to complementary DNA (cDNA), preparing the sequencing library, and sequencing it on

an NGS platform. The methods described below were used for the RNA-sequencing reported in this thesis.

RNA extraction

RNA was extracted from biopsies stored in RNALater (Ambion) at -80C. Biopsies were removed from their storage solution and placed with 1 ml Lysis Buffer (Ambion) in a MK28-R grinder tube (Bertin Instruments) and lysed using a Precellys 24 homogeniser (Bertin Instruments). Tubes were subsequently centrifuged at 1500 xg for 4 minutes, the supernatant removed and the RNA extraction performed using a pure link RNA mini kit (Ambion) as per manufacturer's instructions. Contaminating DNA was removed using TURBO DNase (Ambion) as per manufacturer's instructions. Concentration of RNA was assessed using a Nanodrop Spectrophotometer (Thermo Scientific). Quality of RNA was assessed using a RNA nano Bioanalyzer kit (Agilent) using a Bioanalyzer 2100 (Agilent).

RNA sequencing

1ug of RNA was used for producing libraries for sequencing using TruSeq Stranded total RNA library prep kit (Illumina) as per manufactures instructions with a final PCR amplification of 14 cycles. Libraries were then sequenced on a Hiseq sequencer (Ilumina) by Eurofins.

RNASeq analysis

Following sequencing data was demultiplexed to give individual fastq files using Casava (Illumina). Fastq files were assessed for quality control purpose using FASTQC. The Fastq files were aligned to the human genome (Hg38) using Hisat2. All further analysis was carried out using the R statistical environment. A table of gene counts was produced using the featureCounts function within Rsubread and normalisation and differential gene expression analysis was carried out using DESeq2. For GSEA genes were ranked by the inverse of the p value with the sign of the log fold change and then ran against the hallmarks database within MSigDB using the GSEA program from the broad with the pre ranked option.

CHAPTER 3

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Identifying potential predictive biomarkers of successful ex-vivo lung perfusion

Chapter 3. Identifying potential predictive biomarkers of successful Ex-vivo Lung Perfusion

This chapter describes our investigation into identifying potential predictive biomarkers of successful EVLP in donor lungs that were assessed in the DEVELOP-UK cohort with the intent for transplantation. The multicentre study, *An Observational Study of Donor Ex Vivo Lung Perfusion in UK lung transplantation: DEVELOP-UK* (DEVELOP-UK) (ISRCTN 44922411), was a three-year prospective non-randomised nationwide clinical trial comparing standard lung transplantation with transplantation following lung assessment with EVLP. DEVELOP-UK was launched in 2012 across the five UK lung transplant centres, with the Freeman Hospital (Newcastle) being the lead centre. During the trial, 53 EVLP assessments were performed with 18 of those proceeding to clinical lung transplantation. In each set of lungs, a lung biopsy and standardised research BAL were taken before and after the EVLP assessment and additionally, perfusate samples were collected every 30 minutes during the assessment, **Figure 2.7**. The same sample collection was completed at the other four UK lung transplant centres, and at the end of the study, all samples were transferred to Newcastle for storage and further evaluation. The samples were stored in an HTA licenced and monitored -80 °C freezer at Newcastle University and were available for analysis of a range of biomarkers of lung injury, and inflammation. The collection and storage of samples was part of the DEVELOP-UK study and allowed for complementary mechanistic studies investigating cellular and molecular mechanisms of donor lung reconditioning to be carried out in the future.

To further the understanding of the biology of clinically assessed EVLP lungs a collaboration was initiated with the Transplant Molecular Biology Group at the University of Cambridge specialising in transcriptome analysis. Lead by Dr Menna Clatworthy and Dr John Ferdinand, RNA sequencing analysis was carried out to assess the changes in gene signature within snap frozen tissue from ten human lungs assessed in the DEVELOP-UK study. Samples were obtained at the start and end of EVLP for each lung. The cohort contained sets of lungs that were transplanted (n=6) and declined (n=4) for transplantation based on physiological parameters.

This study aimed to utilise the method of RNA sequencing to determine the genes differentially expressed pre- and post-EVLP, and decipher the corresponding immunological processes. The study also sought to compare the transcriptome between lungs that were transplanted and declined for transplantation in the baseline

samples obtained prior to EVLP. Following up on the interesting findings from the transcriptomic analysis, a further study was pursued to correlate the changes in gene expression with the expression of their corresponding proteins within perfusate.

This chapter reports on the identification of potential biomarkers for organ suitability and the analysis of the corresponding lung perfusate to determine if the proteins associated with the genes identified are detectable and have any predictive value as markers of EVLP performance.

My contribution, in collaboration with our Cambridge colleagues, was to evaluate and analyse the data, correlate with clinical indices and write a manuscript that is to be submitted for publication.

Introduction

Lung transplantation is now established as an appropriate and effective treatment for selected patients with life-threatening end-stage lung disease. Unfortunately, a worldwide shortage of suitable donor organs continues to be the major limitation to the increased accessibility of lung transplantation for those who will benefit. Demand for lung transplantation exceeds the availability of donor organs, and many potential lung transplant candidates do not survive the wait for a suitable donor organ[13].

One common strategy used by transplant professionals to address the challenge of donor shortage is to increase the donor organ pool by increasing the utilisation of lungs from extended criteria donors[13, 123]. These are donors that don't fully satisfy the characteristics of an ideal lung donor by virtue of age, smoking history, lung function or co-morbidity[22-24, 47, 124, 125]. The continual push to use more extended criteria donors has raised concerns among both transplant teams and patients as to whether this approach will lead to poorer early and late outcomes after lung transplantation[126, 127]. One potential solution to this problem is the use of ex-vivo lung perfusion (EVLP) as a means to test the function of extended criteria donor lungs before a decision on suitability[27, 65, 128].

Ex-vivo lung perfusion has emerged over the last ten years as a promising technique to objectively assess and potentially recondition donor lungs unsuitable for immediate transplantation with the overall aim of increasing the available donor lung pool[129]. The decision to accept organs for transplantation after EVLP is currently based predominantly around physiological parameters such as oxygenation, lung compliance, pulmonary vascular resistance and peak airway pressure in addition to the visual appearance of the organ. Reported discard rates of 10–60% of perfused lungs suggest that some donor lungs may be inappropriately used or inappropriately declined for transplant after EVLP[85].

The cellular and molecular events that occur in the donor lung during EVLP have not yet been fully characterised, and there is a pressing need to identify potential predictive biomarkers of inflammation and tissue injury during EVLP to distinguish donor lungs that can be reconditioned and to predict post-transplant outcomes. Our group has previously demonstrated the feasibility of identifying pro-inflammatory and tissue injury signals in perfusion fluid during EVLP of unsuitable donor lungs[83]. Furthermore, we have recently demonstrated that early pro-inflammatory signals in perfusate due to interleukin (IL-1β) and tumour necrosis factor-α (TNF-α), after as little as 30 minutes of

EVLP can distinguish between lung transplant recipients surviving to hospital discharge and in-hospital mortality post-transplantation[83].

Although utilising biomarkers in perfusate seems to be showing encouraging results, the choice of which biomarkers to evaluate in perfusate is based on previously published literature and therefore makes assumptions and is biased to signals reported in studies of primary graft dysfunction after lung transplantation[43, 130-134].

In this study, we have utilised RNA-Seq technology to generate an unbiased profile of the changes in the entire transcriptome of the human donor lung tissue during EVLP. A comprehensive investigation of the changes in tissue RNA expression during EVLP of human donor lungs has not previously been carried out. Our aim with this approach is to detect significant changes in transcriptional activity that might uncover potential biomarkers yet to be identified as playing a significant role in donor lung injury and tissue damage[135].

Methods

Study subjects and protocol

In this study, we utilised samples of whole donor lung tissue collected from a large cohort of highly characterised clinical EVLP procedures performed as part of the DEVELOP-UK multicentre trial, which involved all five UK transplant centres[98]. DEVELOP-UK included 53 adult donor lungs which were deemed unsuitable for lung transplantation but met pre-defined criteria for EVLP. Assessments were performed using a Vivoline LS1 EVLP circuit (Vivoline Medical AB, Lund, Sweden) following 1 of 2 standardised perfusion protocols: an initial Hybrid protocol featured an open left atrium, acellular perfusate, and perfusate flow limited to 40%–60% of donor calculated cardiac output *(n=22)*. Subsequently, the perfusion strategy was changed to the Lund protocol with cellular perfusate (haematocrit 10%–15%) and full flow perfusion of 100% donor calculated cardiac output *(n=31).* All tissue samples were from the Lund protocol. Assessment methods, including sampling procedures, remained unchanged between the two protocols.

Sample collection

The DEVELOP-UK sampling protocol was followed to allow for tissue biopsy collection from either the right middle lobe or lingula both before and after the EVLP assessment using a GIA surgical stapler[98]. Biopsies were snap frozen in liquid nitrogen as soon as possible after collection for subsequent RNA isolation.

A control perfusate sample was collected from the primed EVLP circuit before donor lung perfusion started. Repeated perfusate samples (5 ml) were then collected at 15 and 30 minutes after perfusion commenced and every 30 minutes thereafter. The perfusate samples were centrifuged at 180 xg for 6 minutes at 4^{o}C to remove cellular debris and aliquoted equally into 5x1 ml tubes before being frozen initially at -20^{o}C and then transferred to a -80^{o}C for longer term storage and subsequent laboratory analysis.

RNA extraction

RNA was extracted from biopsies stored in RNALater (Ambion) at -80C. Biopsies were removed from their storage solution and placed with 1 ml Lysis Buffer (Ambion) in a MK28-R grinder tube (Bertin Instruments) and lysed using a Precellys 24 homogeniser (Bertin Instruments). Tubes were subsequently centrifuged at 1500 xg for 4 minutes, the supernatant removed and the RNA extraction performed using a pure link RNA mini kit (Ambion) as per manufacturer's instructions. Contaminating DNA was removed using TURBO DNase (Ambion) as per manufacturer's instructions. Concentration of RNA was assessed using a Nanodrop Spectrophotometer (Thermo Scientific). Quality of RNA was assessed using a RNA nano Bioanalyzer kit (Agilent) using a Bioanalyzer 2100 (Agilent).

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Enzyme-linked immunosorbent assays

Serial perfusate samples from 44 human donor lungs undergoing clinical EVLP assessments were analysed retrospectively. Enzyme Linked Immunosorbent Assays (ELISA) were used to detect protein levels in the perfusate for specific pro-inflammatory markers, including Monokine induced by gamma interferon (MIG), Macrophage Inflammatory Protein-3 beta (MIP-3 beta), Pentraxin-3 (PTX3), Human Alpha-1-acid (α_1AGp) and Heat Shock Protein 70 (HSP-70) (R&D Systems, Inc., Minneapolis, MN). All protein concentrations measured in perfusate were adjusted to the donor predicted total lung capacity as an estimate of perfused donor lung volume and reported as corrected perfusate concentrations (pg/ml).

Statistical Analysis

Perfusate protein levels were analysed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). The ability of each biomarker to discriminate between outcome groups (Survival, Non-survival, and Decline) was assessed with Mann-Whitney U test.

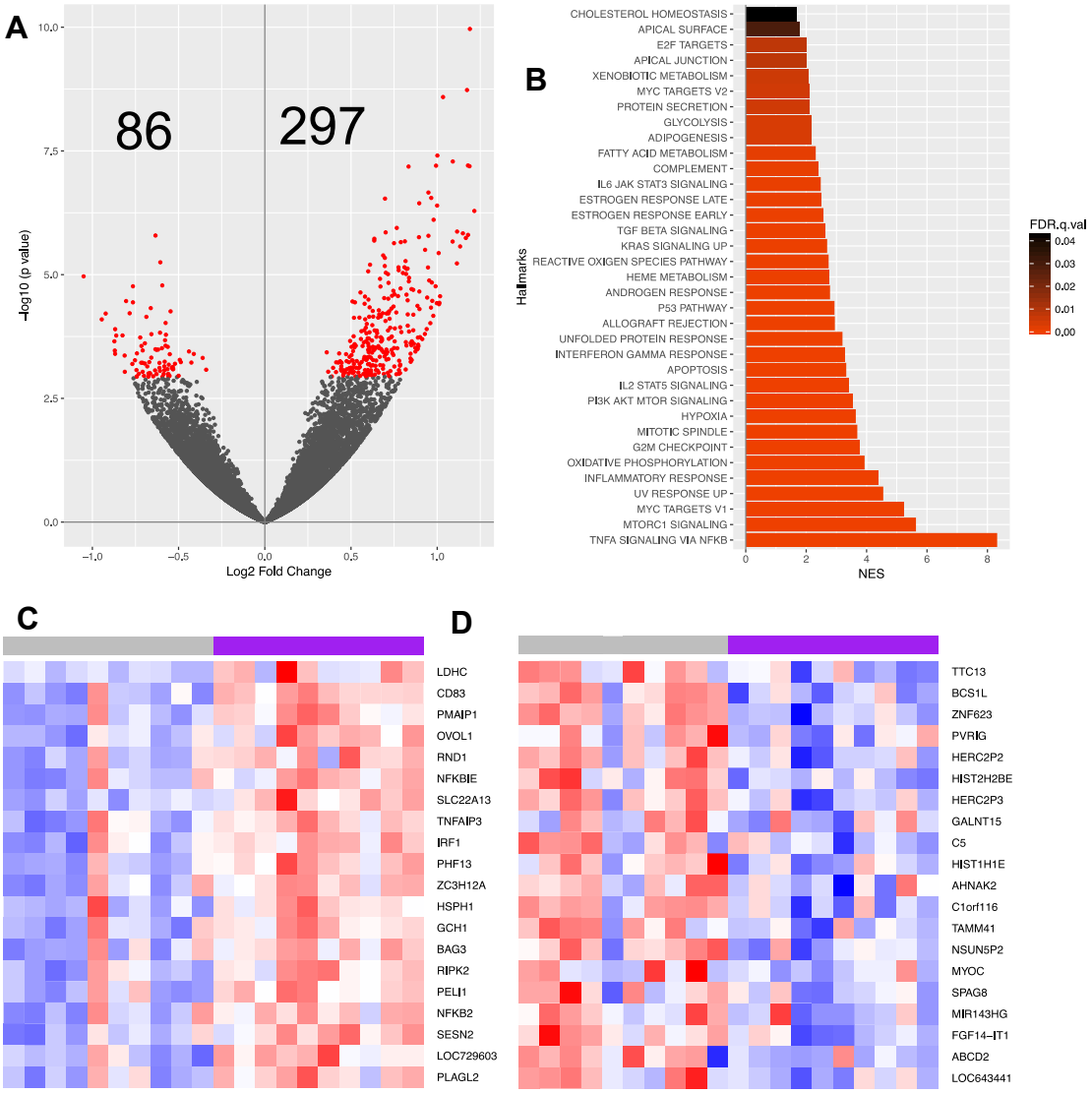
Results

Between April 2012 and July 2014, 53 donor lungs, deemed unsuitable for immediate transplantation underwent EVLP as part of the DEVELOP-UK clinical trial. A detailed description of the study population can be found in the final study report[98]. Due to incomplete or insufficient sampling, complete perfusate samples were available from 44 of the 53 donor lungs and both pre- and post-EVLP tissue samples from 10 of the 53 donor lungs. Of the 44 donor lungs included, 18 (41%) underwent "successful EVLP" and met the criteria for transplantation, while a further 4 of those 18 met transplant criteria but did not proceed to transplantation due to logistical or donor reasons. 26 of 44 donor lungs (59%) did not satisfy criteria for transplant after EVLP. Of the 14 lung transplants that were carried out following EVLP, 9 of 14 recipients (64%) survived to 1-year. The median donor age was 49.5 years (range 16-65 years). There were 25 male and 19 female donors. There were 33 DBD donors and 11 DCD donors. The results were compared between sub-groups depending on outcome: Successful EVLP n=18, Unsuccessful EVLP n=26. The results were also compared between sub-groups depending on the EVLP protocol used: Hybrid n=15 and Lund n=29.

In this study we compared the gene expression signatures in paired pre and post EVLP lung tissue samples to determine the changes in gene expression that occur during the process of EVLP. We studied the RNA-Seq profiles from 10 EVLP procedures in total. We further categorised them depending on whether the EVLP was deemed successful ("pass" n=6 or unsuccessful "fail", n=4) using the standard donor lung assessment criteria defined used as part of the DEVELOP-UK trial[98]. Comparisons of the genetic signature in these two groups was then carried out.

Our results illustrated that the process of EVLP alone induce a change in the gene expression within donor lung tissue**, Figure 3.1.** Perfusion itself causes changes within the lung transcriptome, predominantly pathways which are involved in the immune system. The effect of perfusion on the transcriptome is demonstrated in the volcano plot, **Figure 3.1A** showing that EVLP was associated with the significant upregulation of 297 and down regulation of 86 specific genes. During EVLP several cytokine genes were significantly upregulated **Figure 3.1F**, with CXCL5, TNF, CCL20 and CXCL1 all showing significance (padj <0.05). During the process of EVLP there are also a number of genetic pathways that are upregulated. The gene pathway that was most significantly upregulated was TNFA Signalling via NFκB. Other important pathways

that were highly upregulated were hypoxia, inflammatory response and oxidative phosphorylation **Figure 3.1C**.



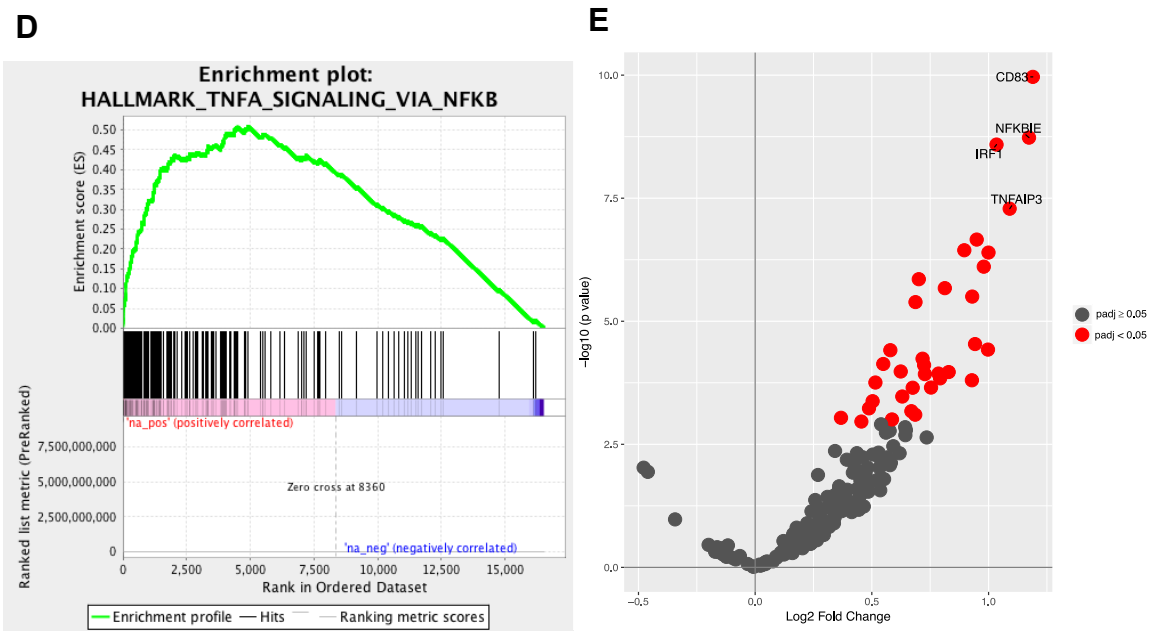


Figure 3.1 EVLP induces a large effect on the transcriptome which is predominated by immune pathways

Lung tissue biopsies were collected before and after each EVLP assessment, n=10. Biopsies were snap frozen in liquid nitrogen for subsequent RNA extraction.

A. shows a Volcano plot for the effect of perfusion. Number indicate the total number of significant genes in each direction: 86 down regulated and 297 upregulated during perfusion.

B. shows a gene set enrichment analysis (GSEA) against the hallmark data set for perfusion demonstrating that the TNF-α signaling via NFκB pathway is the gene set most highly expressed.

C. shows the heat map of the top 20 genes up and down regulated during perfusion with pre EVLP samples shown in the grey panel and post EVLP samples shown in purple panel. Range of colours (red to blue) shows the range of expression values (high to low).

D. shows the enrichment plot for the gene set TNF-α signaling via NFκB by GSEA. Bottom, plot of the ranked list of all genes. Y axis, value of the ranking metric; X axis, the rank for all genes. The enrichment plot demonstrates that with a peak enrichment score of 0.50, the TNF-α signaling via NFκB gene set is ranked at the top of the list of genes found from the GSEA.

E. shows a volcano plot for the effect of perfusion on cytokine genes

We demonstrated that there was a direct correlation between the cytokine level in perfusate with the RNA count prior to commencing perfusion, **Figure 3.2**.

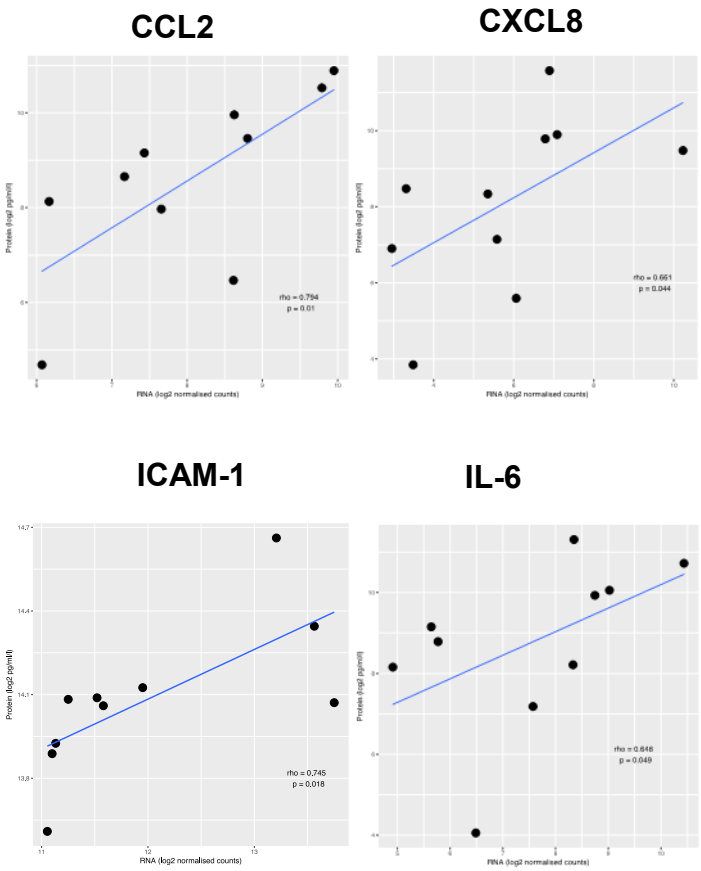


Figure 3.2 Cytokine levels in the perfusate correlate with the RNA count in pre EVLP lung tissue samples.

Scatter plots show the correlations between the cytokine protein level (log2 pg/ml/l) in perfusate (Y-axis) and the RNA count (log2_normalised counts) for the corresponding cytokine gene detected in the pre EVLP lung tissue (X-axis). Correlation is for untransformed data. Liner regression line added to log transformed data. CCL2, P=0.01; CXCL8 P=0.044; ICAM-1 P=0.018; IL-6 P=0.049.

The assessment of the transcriptome demonstrated differences in the gene profiles between donor lungs that performed well during EVLP (pass) compared with donor lungs that performed poorly and could not be transplanted (fail). Gene set enrichment analysis (GSEA) was used to identifying significantly enriched gene sets in pass and fail lungs. There is increased induction of immune activation genes and pathways during perfusion in fail compared to pass lungs. **Figures 3.3 and 3.4** show that in lungs which had a poor outcome, there was an upregulation of immune pathways such as interferon gamma response and TNF via NFκB. Conversely the oxidative phosphorylation pathway was downregulated in fail lungs.

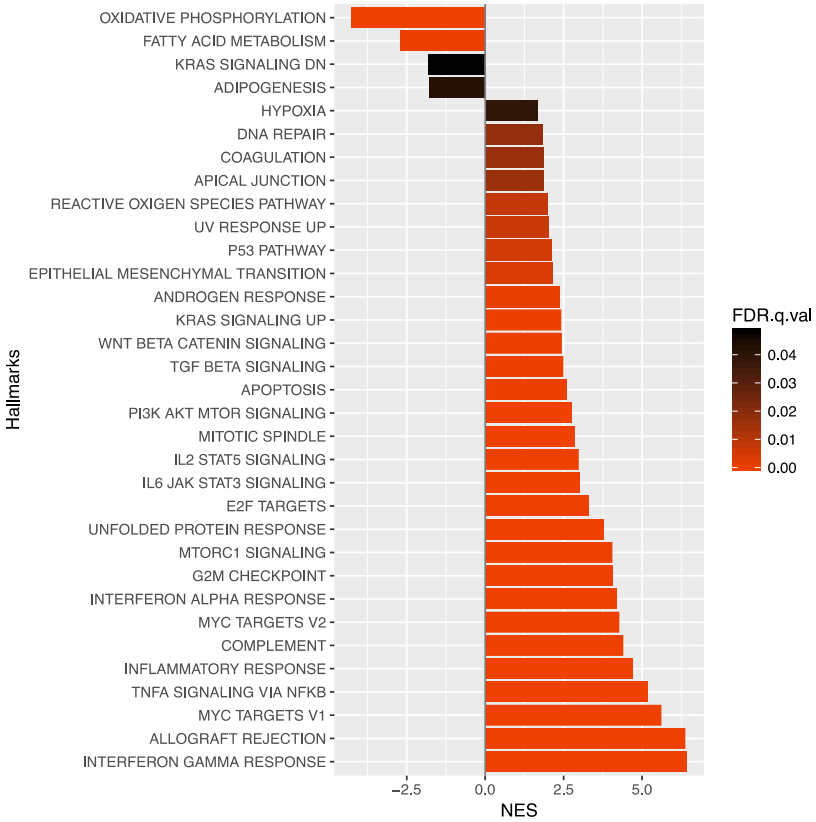


Figure 3.3 There are differentially expressed gene sets in lung tissue from pass versus fail EVLP.

Lung tissue biopsies were collected before each EVLP assessment. They were then divided into pass or fail EVLP depending on whether they met Transplant criteria at the end of EVLP, "pass" n=6 and "fail", n=4. For fail lungs the hallmarks GSEA for interaction can be seen above.

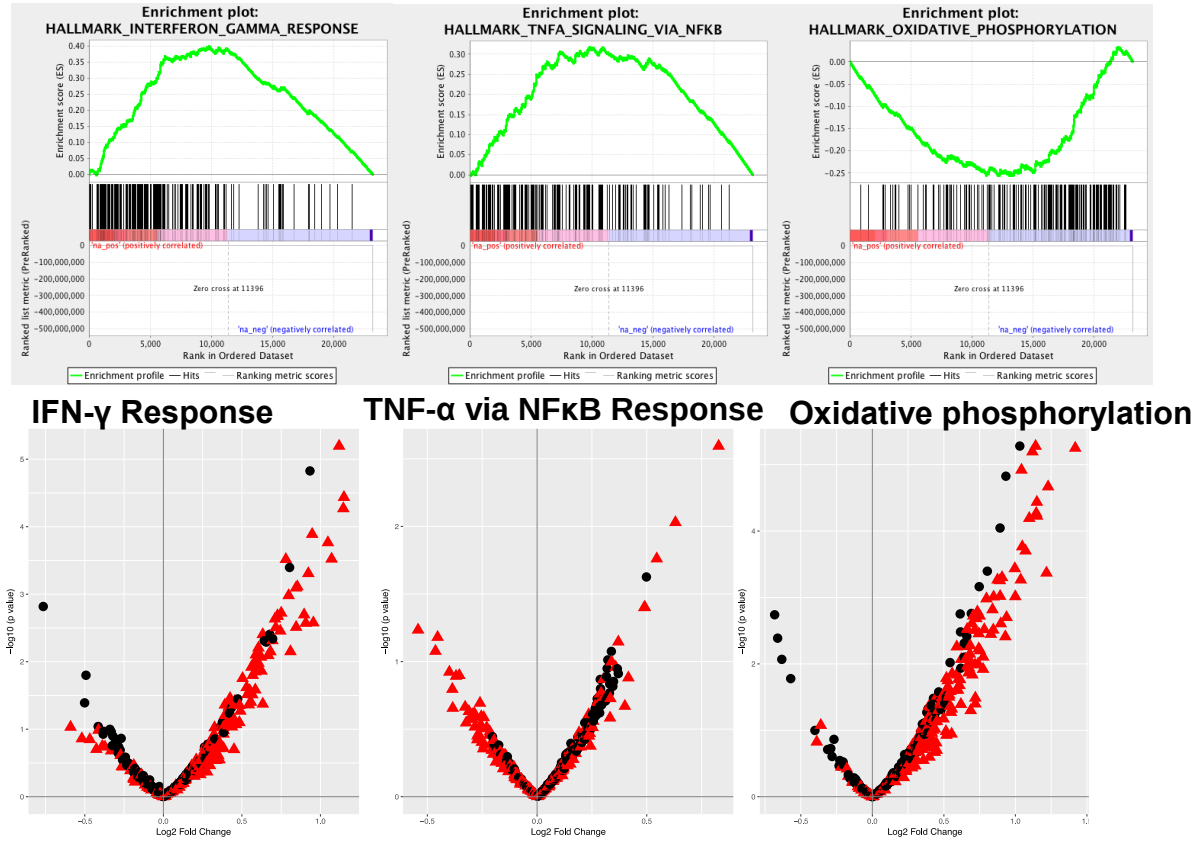


Figure 3.4 There are differentially expressed gene sets in lung tissue from pass versus fail EVLP.

Top row shows the GSEA enrichment plots for the interferon gamma, TNF-α via NFκB and oxidative phosphorylation responses.

Bottom row shows the volcano plots for the individual genes within each pathway from GSEA analysis in row above. Red triangle points are the lungs which failed EVLP, black circle points are those that passed. The Log fold change is for the comparison within groups between pre and post samples.

IL-1β is a powerful pro-inflammatory cytokine that is a hallmark of sterile inflammation occurring as a result of NLRP3 inflammasome activation. We demonstrated that inflammasome activation genes are enriched in pre-perfusion biopsies in fail lungs, **Figure 3.5**.

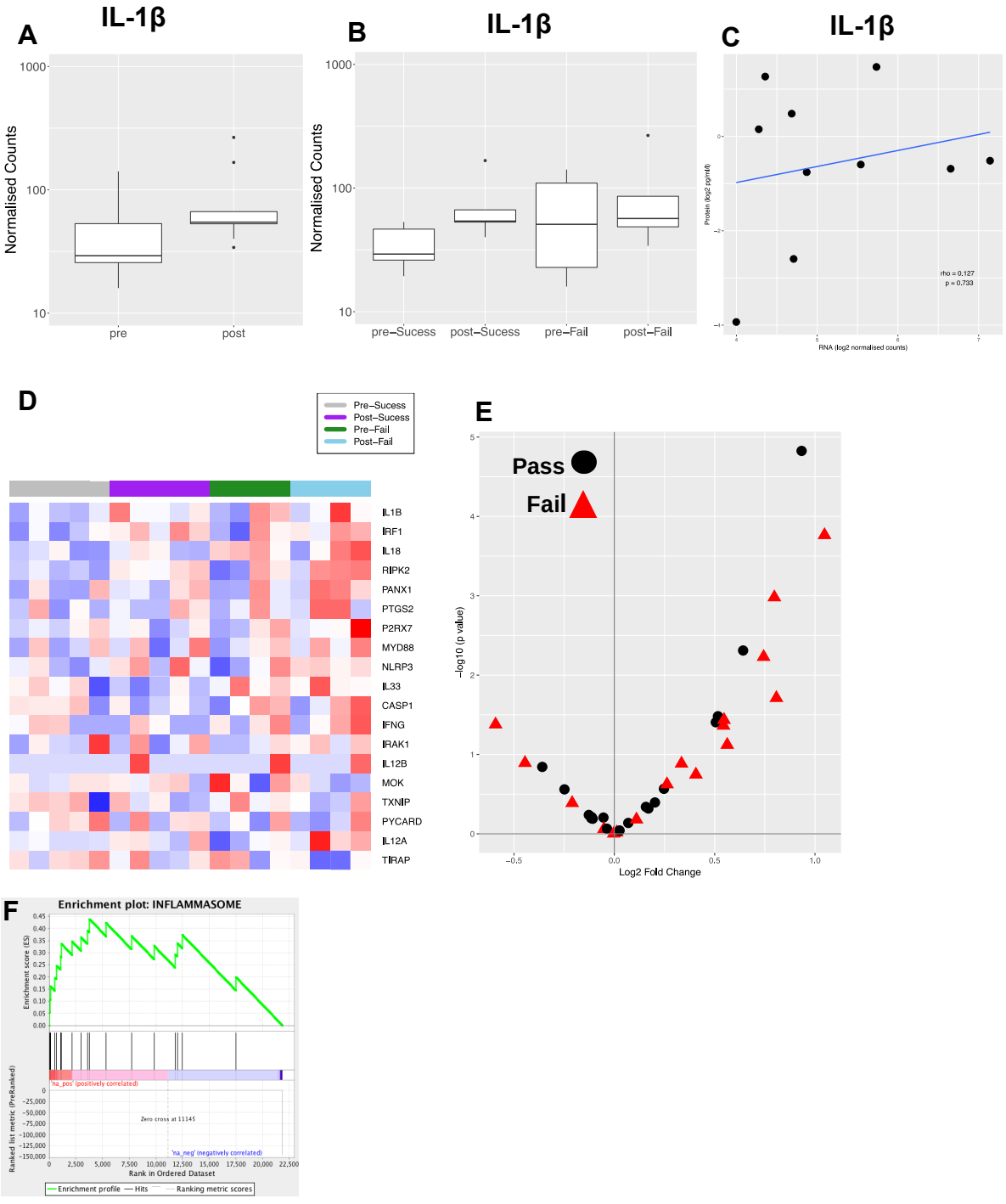


Figure 3.5 The effect of EVLP and outcome on IL-1β and the inflammasone genes

A. shows the normalised counts of IL-1β for perfusion in all samples between pre and post perfusion. Higher IL-1β counts seen in the post samples.

B. shows the normalised counts split by time point and outcome. Prior to commencing perfusion, the lungs that go onto later fail have higher normalised counts of IL-1β.

C. shows the correlation for the IL-1β RNA level in samples pre perfusion with the protein content in the perfusate, P=0.733.

D. shows a heat map of the inflammasone related genes. All individual cases are seen: pre samples for the pass group shown in the grey panel; post samples of the pass group in the purple panel; pre samples for the fail group in the green panel and post samples for the fail group in the blue panel. This demonstrates that the inflammasone genes are upregulated during EVLP, and significantly more in lungs that fail.

E. shows the GSEA of inflammasone gene in pre EVLP samples comparing pass versus fail, P=0.004. Inflammasone genes are significantly upregulated in pre samples of fail lungs.

F. shows the Volcano plots for the individual genes within each pathway from GSEA analysis in D. Red triangle points are the lungs which failed EVLP, black circles are those that passed. The Log fold change is for the comparison within groups, between pre and post samples.

Gene transcripts of all neutrophil-recruiting chemokines were evaluated and showed that lungs that failed during EVLP express higher level of CXCL8 mRNA and have a trend towards a greater increase during perfusion and a trend towards higher CXCL8 mRNA levels in post-perfusion samples, **Figure 3.6**.

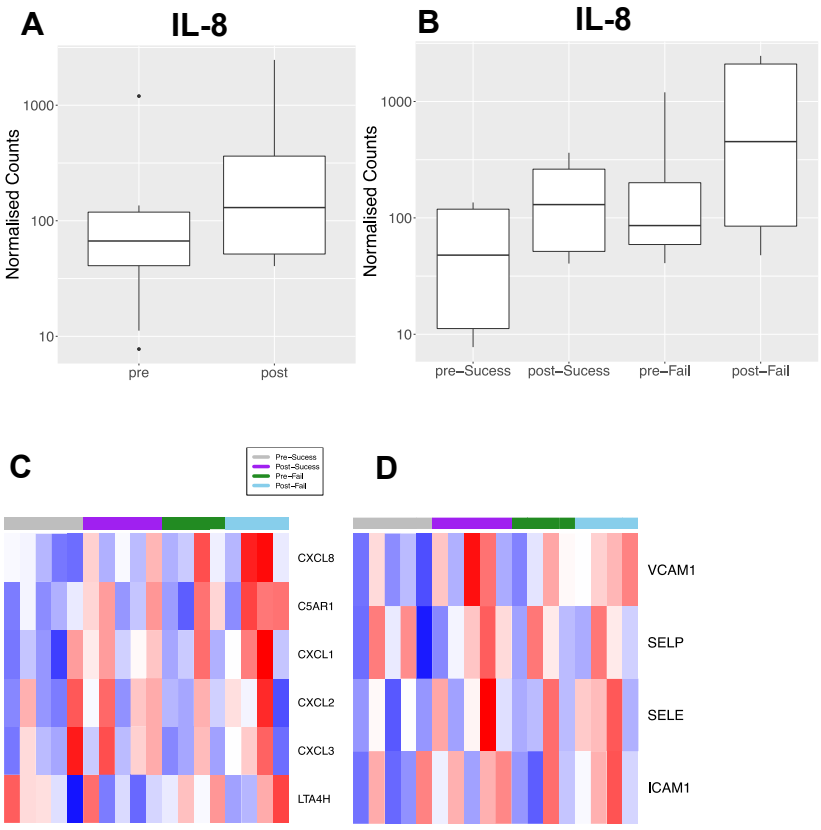


Figure 3.6 The effect of perfusion and outcome on the IL-8 gene set.

A. and B. show the normalised counts for CXCL8 in the indicated comparisons. Higher IL-8 counts seen in the post samples. Prior to commencing perfusion, the lungs that go onto later fail have higher normalised counts of IL-8.

C. shows a heat map of neutrophil chemo attractants.

D. shows a heat map of neutrophil related adhesion proteins.

A subset of heat shock proteins is upregulated during perfusion in fail lungs, **Figure 3.7. Figure 3.8** demonstrates the changes in cytokine and chemokine gene expression when comparing pass and fail lungs, pre and post EVLP.

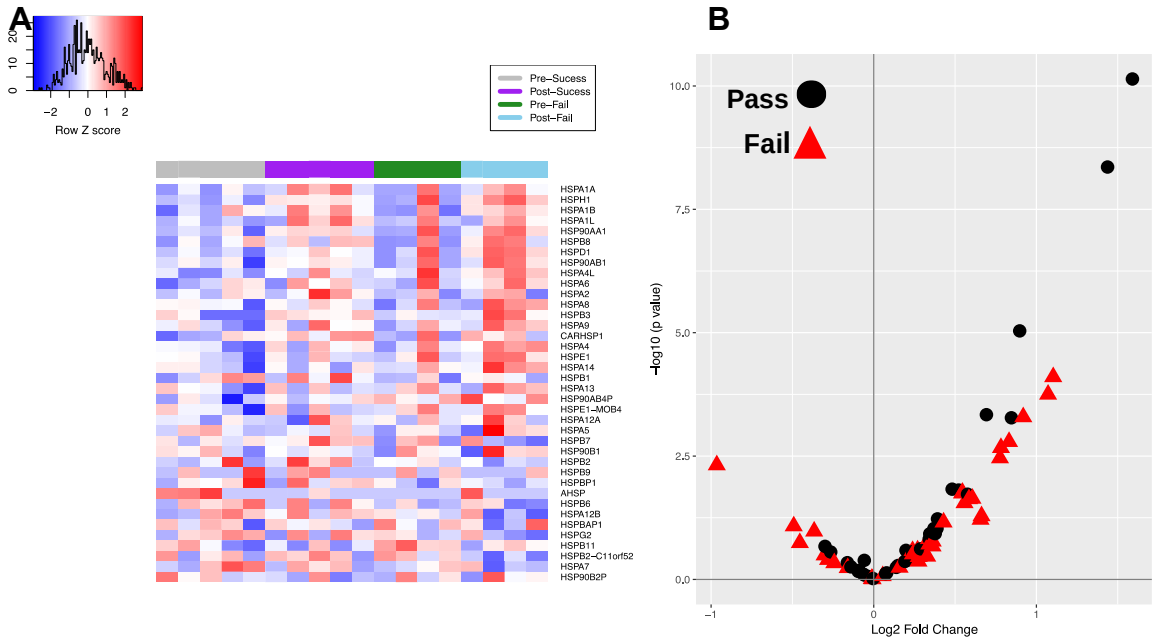


Figure 3.7 Heat shock protein family genes are seen in higher levels in lungs that fail EVLP

A. *Heatmap of HSP family of proteins – many of these are increased following perfusion in samples which fail EVNP.*
B. *Volcano plot of genes above for effect in perfusion split by outcome group.*

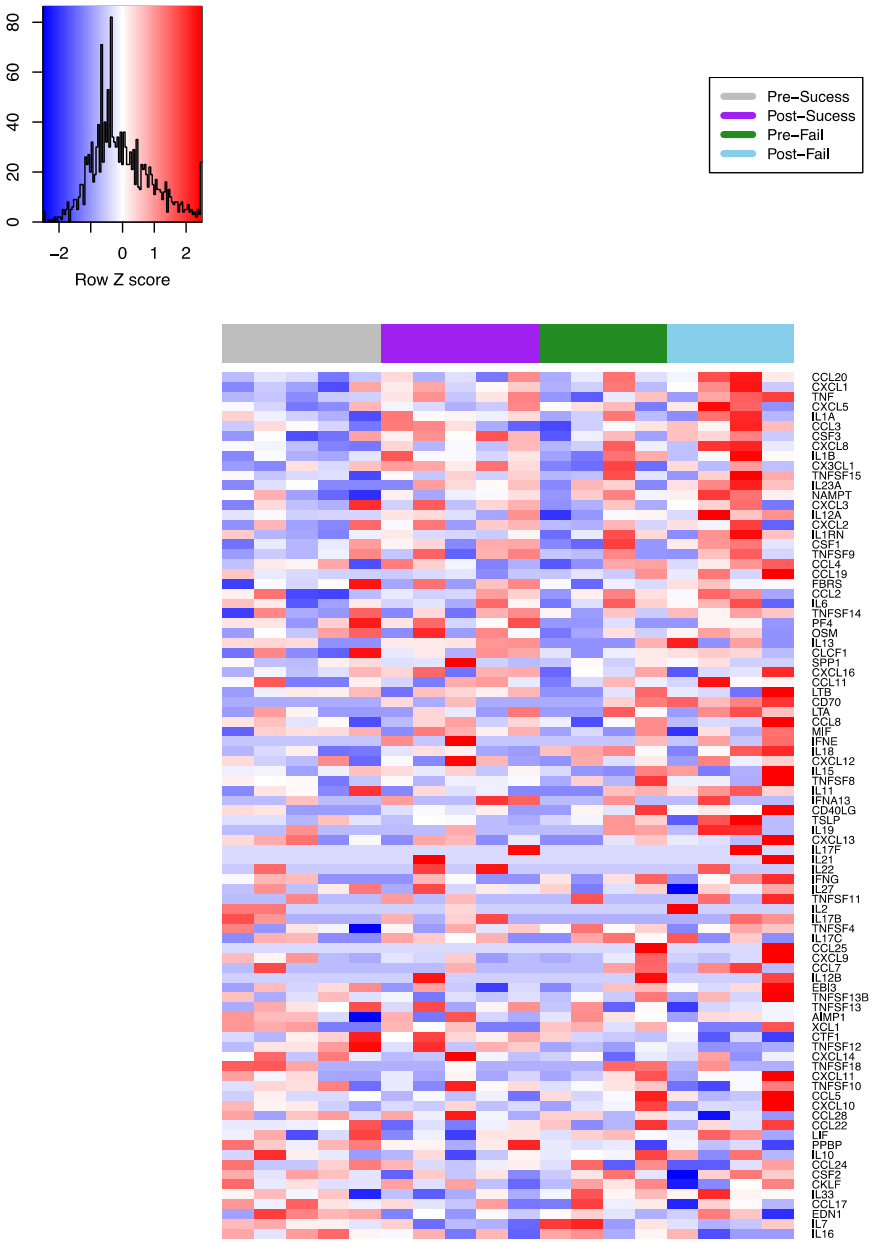


Figure 3.8 Heat map of cytokines and chemokines between pass and fail EVLP lungs

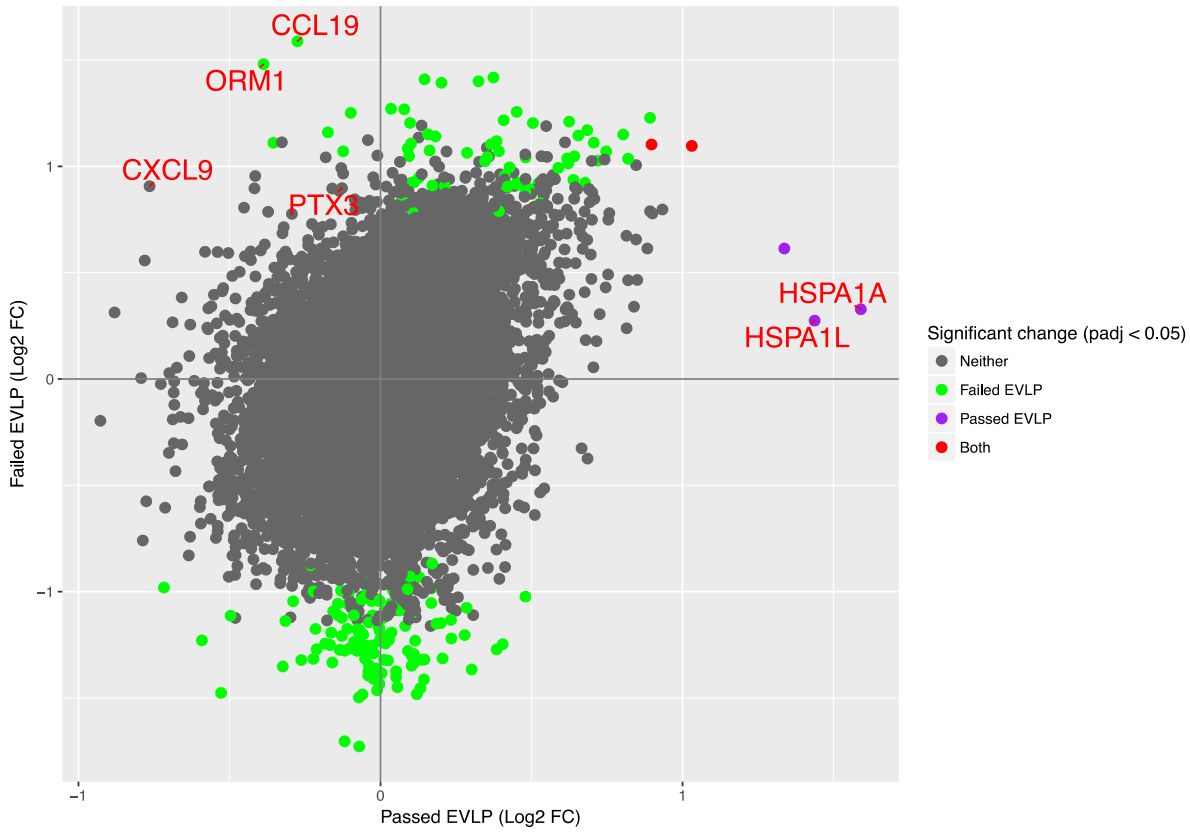


Figure 3.9 Potential biomarkers in EVLP

A volcano plot showing the logarithmic fold change (LFC) between the outcome groups with potential biomarkers highlighted in red, indicates which genes taken forward for protein validation. In lungs that passed EVLP, there were significantly higher levels of the genes for HSPA1A and HSPA1L. CCL19 and ORM1 were upregulated in lungs that failed EVLP.

Perfusate

Following this unbiased approach of identifying genes, **Figure 3.9,** that were only upregulated in fail lungs with poor outcome, we found that there were 4 genes significantly upregulated in only fail lungs and that these genes corresponded to pro-inflammatory cytokines MIG and MIP-3β; the acute phase proteins PTX3 and α_1AGp. In lungs that passed EVLP, there were significantly higher levels of the genes for HSPA1A and HSPA1L. We then investigated the protein expression in perfusate for these specific pro-inflammatory and tissue injury proteins of interest. We then compared the levels of cytokine expression between the clinical outcome and protocol groups to assess if we could determine any clinical correlations.

The results were compared between sub-groups depending on outcome: Successful EVLP (pass) n=18, Unsuccessful EVLP (fail) n=26. The results were also compared between sub-groups depending on the EVLP protocol used: Hybrid n=15 and Lund n=29.

There were no significant differences in the levels of protein expression for the 5 markers comparing the fail lung group compared with the pass lung group**, Figure 3.10**.

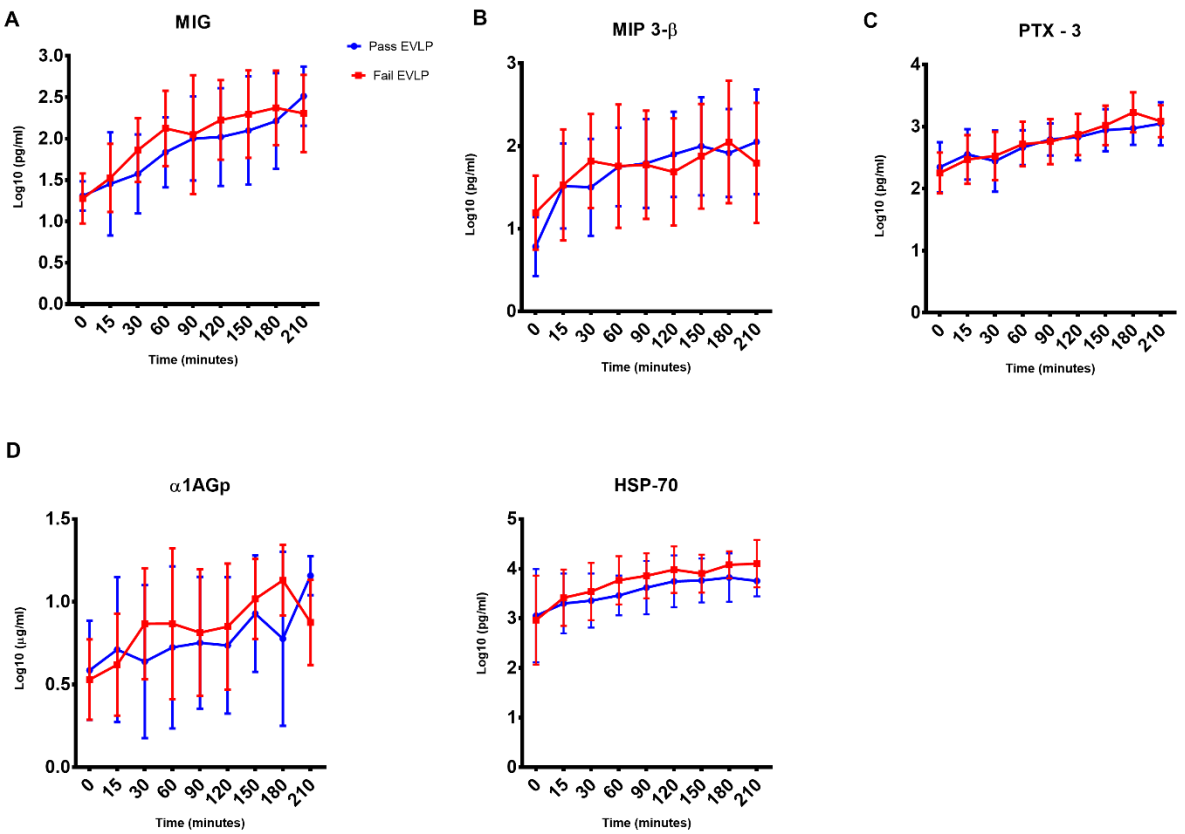


Figure 3.10 Perfusate potential biomarker levels comparing pass and fail outcome groups.

Line graphs demonstrating the logged protein biomarker concentrations measurable in perfusate by ELISA comparing EVLP outcomes: pass (blue) and fail (red) EVLP of donor lungs measurable by ELISA. Logged perfusate levels of A) MIG in pg/ml B) MIP-3β in pg/ml C) PTX-3 in pg/ml D) α1AGp µg/ml and E) HSP-70 in pg/ml over the course of the EVLP assessment separated into outcome groups (mean ± SD), Pass n=18, Fail n=26. There was no significant difference in perfusate expression of any of the 5 protein markers at any EVLP time point in pass vs. fail EVLPs. EVLP: ex vivo lung perfusion; MIG: Monokine induced by gamma interferon; MIP-3 Beta: Macrophage inflammatory protein 3 beta; PTX-3: Pentraxin-3; α1AGp: Human alpha-Acid Glycoprotein; HSP-70: Heat shock protein 70.

When we compared the protein expression of the 5 markers between Hybrid and Lund protocol groups there were statistically significant differences**, Figure 3.11**. There was an increase in MIG perfusate concentrations in EVLPs performed using the Hybrid protocol compared to Lund protocol at all time-points during EVLP. This was detected as early as 15 minutes from commencing EVLP, P1 (Lund: M=31.64pg/mL, Hybrid: M=687.1pg/mL, p=0.0263). This remained significantly different until 210 minutes of perfusion, P8 (Lund: M=247.6pg/mL, Hybrid: M=646.5pg/mL, p=0.0263). There was a statistically significant increase in MIP-3β perfusate concentration at 30 and 60 minutes of EVLP performed using the Hybrid protocol compared to Lund protocol, P2 (Lund: M=53.2pg/mL, Hybrid: M=230.1pg/mL, p<0.0074), P3 (Lund: M=78.17pg/mL, Hybrid: M=272.1pg/mL, p<0.0214). There were no significant differences at any time points for Pentraxin-3, Human alpha-1 acid Glycoprotein or HSP-70.

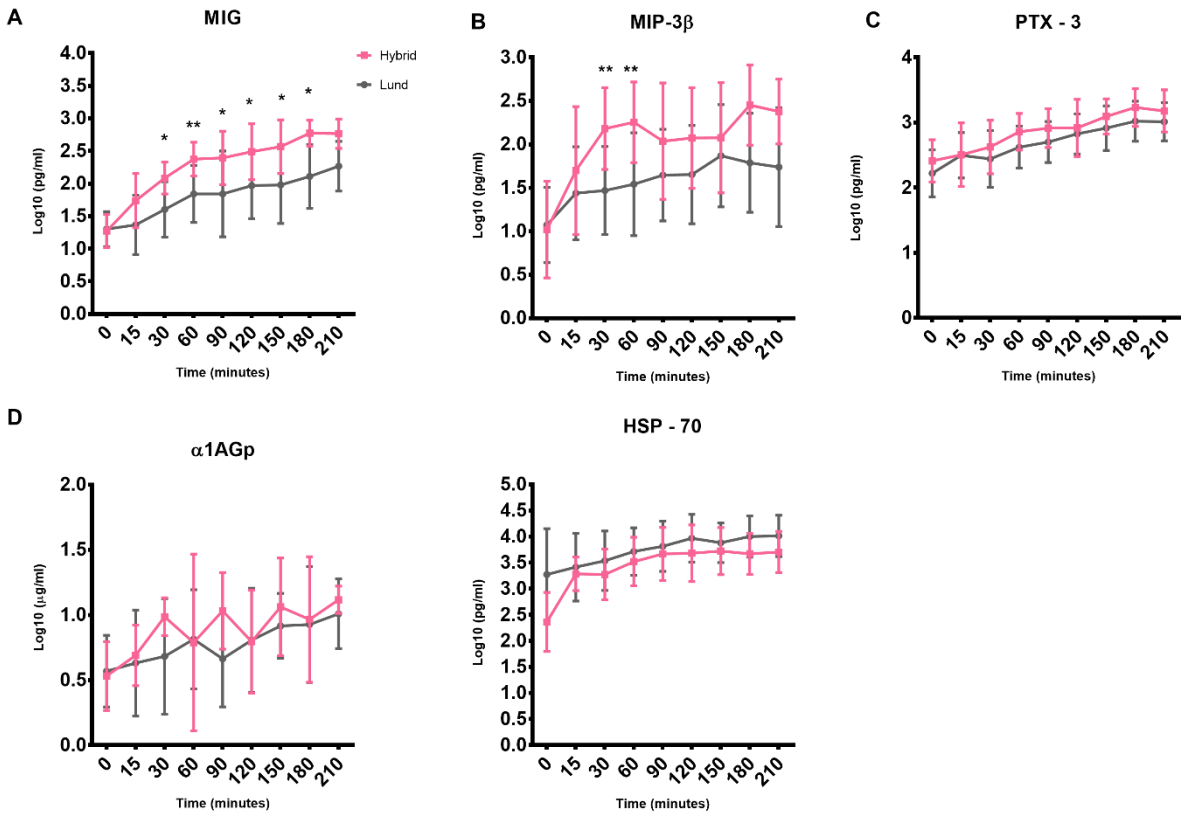


Figure 3.11 Perfusate potential biomarker levels comparing Hybrid and Lund protocol groups.

*Line graphs demonstrating the logged protein biomarker concentrations measurable in perfusate by ELISA comparing EVLP protocols: Hybrid (grey) and Lund (pink) A) MIG in pg/ml B) MIP-3 beta in pg/ml C) Pentraxin-3 in pg/ml D) α1AGp in µg/ml E) HSP-70 in pg/ml over the course of the EVLP assessment separated into outcome groups (mean ± SD), analysed using unpaired t-tests and Bonferroni corrections, *P<0.05. **P<0.01, Hybrid n=15, Lund n=29. EVLP: ex vivo lung perfusion; MIG: Monokine induced by gamma interferon; MIP-3 Beta: Macrophage inflammatory protein 3 beta; PTX-3: Pentraxin-3; α1AGp: Human alpha- Acid Glycoprotein; HSP-70: Heat shock protein 70.*

Discussion

If ex-vivo lung perfusion (EVLP) is to realise its potential as an innovative platform for the assessment and treatment of donor lungs prior to transplantation, it's essential that the cellular and molecular events that occur during EVLP are fully understood. This study presents an in-depth, unbiased analysis of the lung transcriptome in donor lungs not deemed suitable for immediate transplantation and how this changes during the EVLP procedure. We have shown that genes associated with a relatively small number of specific pathways are either upregulated or downregulated during EVLP and that this is affected by both the EVLP protocol followed and the performance of the organ during the objective assessment.

Lungs are highly susceptible to acute injury in the critical care environment. In the hours or days leading up to the donor's demise, they are often exposed to multiple insults including: the sequelae of brain-stem death, infection, aspiration, barotrauma, fluid overload or multiple transfusions[19]. The extent of donor lung injury is difficult to assess at the time of organ procurement, and therefore donor acceptance criteria have historically been conservative and act as poor discriminators of injury and infection in the donor lung[24]. Robust biomarkers that can stratify donor lungs during EVLP as being suitable for transplant or in need of additional targeted interventions would enhance its clinical impact. This study evaluated the feasibility of using lung transcriptomics to determine if specific and significant changes in the genetic profile of the donor lung may reveal novel potential protein biomarkers of lung performance during clinical EVLP.

We have demonstrated that expression of many genes changes by either upregulation or downregulation during the act of perfusion itself. Furthermore, many of the genes and the pathways that are upregulated are immune pathways, including TNF-α signalling via NFκB, cytokine interaction and intracellular signalling pathways. Our analyses also demonstrated that there are differential changes in gene expression occurring during EVLP between lungs which pass and fail the assessment of their transplant suitability.

Andreasson *et al*. demonstrated levels perfusate IL-1β is a promising predictor of successful EVLP as well as the post-transplant outcome during clinical perfusion of extended criteria donor lungs, demonstrating a measurable increase in perfusate IL-1β at 30 min in donor lungs *Declined* compared to those transplanted. A TLC scaled cutoff value of 0.1pg/ml, perfusate IL-1β following 30 min of EVLP had a sensitivity

and specificity of 100% to diagnose *Non-survival* lungs and could be used to distinguish 1-year survival with similar precision. Perfusate interleukin-1β levels were strongly associated with early graft performance and could in the DEVELOP-UK multicentre study with high sensitivity and specificity diagnose both EVLP assessment failure and early post-transplant mortality. This study showed that inflammasome activation genes are enriched in pre-perfusion biopsies in fail lungs. Figure 4D shows that in lungs that went on to fail, there was clearly upregulation of the IL-β genetic signal prior to commencing perfusion.

Our study showed that genes associated with inflammasome activation are enriched in pre-perfusion biopsies in lungs that fail EVLP assessment. Increased levels of the neutrophil chemokine interleukin IL-8 in the lungs of patients who have suffered severe trauma have been shown to predict subsequent development of acute respiratory distress syndrome. The lungs of brain-dead organ donors can contain high levels of IL-8. Fisher *et al.* investigated whether this may predispose to early graft failure in the recipient after lung transplantation and found that that increased levels of IL-8 in the airspaces of a donor lung are associated with the development of severe primary graft dysfunction (p 0.027) and with early recipient mortality (p 0.0034)[43]. Since IL-8 is a neutrophil-recruiting chemokine, we analysed transcripts of all neutrophil-recruiting chemokines to see if these were increased in lungs that failed to improve at the start of perfusion. This shows that lungs that fail tended to start with a slightly higher level of CXCL8 transcripts and trended towards a greater increase during perfusion resulting in higher CXCL8 levels in post-perfusion samples.

The activation of pro-inflammatory cytokines such as IL-1β has been shown to correlate to an increase in cell adhesion molecules and the infiltration of leukocytes[83]. Our pathway analysis demonstrated the enrichment of gene sets involved in both cell communication and adherens junctions. The upregulation of these pathways highlights the role that endothelial barrier integrity plays in the pathophysiology of ischaemia reperfusion injury and ultimately membrane permeability and EVLP success.

RNA-seq allowed us to identify genes up-regulated in either pass only or fail only donor lungs without bias whose associated protein product might be considered to be potential biomarkers for predicting EVLP outcomes for validation and clinical correlation. Our results demonstrate significant differences in perfusate protein expression between lungs that passed EVLP and were deemed transplantable

compared with those that failed. Significant differences were detected when comparing the Hybrid and Lund protocols with a clear separation in MIG and MIP-3β protein expression. We observed a measurable increase in perfusate MIG and MIP-3β levels throughout EVLP for donor lungs that underwent the Hybrid protocol compared with those that underwent the Lund protocol. The hybrid protocol (n=15) combined the open left atrium used by Steen *et al*. and the reduced flow rate of the Toronto method in an effort to reduce pulmonary vascular shear stress and oedema formation and an acellular perfusate. During the DEVELOP-UK study, the Hybrid protocol was abandoned, because of a high rate of post-operative ECMO, to the Lund protocol for the remainder of the study (n=29) keeping the left atrium open but changing to a cellular perfusate and 100% cardiac output. While RNAseq demonstrated medium and high levels of expression of Orosomucoid 1 and Pentraxin 3 respectively with levels increasing in declined lungs only, this was not reflected at a protein product level in the perfusate, with very similar levels of PTX-3 observed in both pass and fail lungs and higher levels of α_1AGp identified in successful EVLPs. As the biomarkers show the greatest difference is between the perfusion methods may suggest one protocol is better/ promotes healthier lungs when compared. Further, it may suggest that as we cannot see the effect at the protein level across the data set, an RNA based gene-expression test may be more appropriate.

Immune profiling in lung transplantation has relied primarily on downstream protein production and cellular analysis. If the purpose of the biomarker discovery is to detect the onset of ischaemia reperfusion injury before an uncontrollable immune activation takes place, then as a method of immune profiling, RNA-Seq transcriptomic analysis illuminates not only individual genes, but entire pathways and upstream regulators. This can provide a fuller picture of the underlying pathology and allow for identification of potential upstream targets to prevent injury before the onset of significant inflammation. Several investigators have shown that brain death and ischaemia-reperfusion injury have a significant impact on complex gene expression changes [37, 136, 137].

The sample size of 10 is consistent with that of other gene profile studies in the lung transplantation field[138, 139]. Although small, we importantly were able to use paired samples of lung tissue for analysis. The results of this study need further evaluation in a larger validation cohort of lungs exposed to EVLP but demonstrate

the feasibility of identifying the changes in the lung transcriptome during EVLP and comparing outcome groups.

The study cohort was that of marginal lungs which had been deemed unsuitable for transplantation, and so they reflect a different cohort of donor lungs compared with those of standard criteria.

Our unbiased and pathway focused approach has provided novel insights into the potential mechanisms activated before and after EVLP. Up-regulation of the genes in our EVLP cohort clearly illustrates the onset of an inflammatory environment. Novel upstream pathways associated with this inflammation could provide an early warning mechanism to impending lung injury and rejection and identifiable targets for pharmacologic intervention. Future studies will need to investigate emerging real-time cytokine tests, as time-sensitive assays with high sensitivity will be essential for the utility of any biomarker during clinical EVLP.

CHAPTER 4

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A comparison of inflammatory profiles in donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD)

Chapter 4: A comparison of Inflammatory profiles in donor lungs following donation after brain-death (DBD) and donation after circulatory death (DCD).

In Chapter 3, we demonstrated the feasibility of identifying pro-inflammatory signals in perfusate during EVLP reconditioning of initially unsuitable donor lungs. With access to the large DEVELOP-UK sample cohort, we pursued whether identifying pro-inflammatory signals could help us determine biological differences between DBD and DCD donor lungs.

Given the global shortage of suitable human donor lungs available for transplantation and the associated waiting list morbidity and mortality, one of the main aims of the lung transplant community is to expand the donor lung pool. One of the approaches to achieve this is through the use of organs from donation after circulatory death (DCD) donors. Although there are several studies from multiple centres show comparable clinical outcomes following DBD and DCD donor lung transplantation, there are only a limited number of studies investigating the disparities between these two donor types and their characterisation at a cellular and biological level.

This study aimed to ascertain if there are any differences in the pro-inflammatory profiles and expression of tissue injury markers between lungs from DBD and DCD donors before and during EVLP. We evaluated a panel of inflammatory mediators, tissue injury-associated proteins and angiogenic factors in perfusate, bronchoalveolar lavage fluid (BALF) and lung tissue from human donor lungs undergoing clinical EVLP with intent for transplant. We hypothesised that DBD lungs and their associated systemic inflammatory response following brain stem death would exhibit increased levels of pro-inflammatory and injury markers when compared to DCD lungs from the outset of EVLP.

Sample processing had been previously carried out by PhD student, Mr Anders Andreasson before my arrival, with raw data available for analysis. My contribution was to analyse and evaluate the raw data along with the generating discussion, execution and write up. The results of this study are presented in the original manuscript presently in preparation for journal submission.

Introduction

Although lung transplantation is regarded as a safe and effective therapy for selected patients with end-stage lung disease, the demand for donor organs still exceeds the overall donor organ supply, and many transplant candidates do not survive long enough for a suitable donor to be identified[13]. One of the approaches to expand the donor lung pool is through the use of organs from donation after circulatory death (DCD) donors[15]. Early studies showed that the lung remains viable after circulatory arrest for a period of time[140, 141]. Several studies from both individual institutions and national transplant organisations and registries have reported that the outcomes in controlled DCD lung transplantation are comparable with those from donors after brain death (DBD)[16, 68] [142-147] A meticulously performed meta-analysis of five studies found no difference in short or long-term survival, between transplants performed with DCD or DBD donors[71]. Only two reports have demonstrated worse outcomes with respect to primary graft dysfunction (PGD) and bronchiolitis obliterans syndrome (BOS)[143, 148]. The use of DCD donors varies across the globe, accounting for less than 5% of lung transplant activity in the US[69][149]compared with 20% in the UK[13].

Brain death itself leads to hemodynamic, metabolic, and neuroendocrine abnormalities in the donor resulting in radiological and physiological abnormalities in the lungs or so-called neurogenic pulmonary oedema[19]. Brain death generates a systemic inflammatory response with the release of key pro-inflammatory mediators, leading to upregulated expression of adhesion molecules on pulmonary endothelium and subsequent leukocyte tissue infiltration[19] Patients suffering irreversible catastrophic brain injury, resulting in a diagnosis of brainstem death, have a high incidence of associated pulmonary dysfunction and a much higher expression of neutrophil chemokines in their lungs than non-brain dead ventilated controls [43]. This initial insult in combination with possible airway aspiration, respiratory tract infection, atelectasis and pulmonary contusion, may all contribute to lung damage before donation occurs [85]. Previous studies have linked the acute inflammatory response seen after brain stem death and an increased risk of PGD and early mortality post transplantation[43].

PGD is a leading cause of early morbidity and mortality after lung transplantation. In just one study, by Sabashnikov *et al.,* there was a higher incidence of PGD and a trend towards greater need for extracorporeal life support (ECMO) in a series of 60 DCD lung transplant recipients compared to their score matched DBD recipients[148].

However, the Krutsinger meta-analysis found no difference in PGD incidence between lungs from DCD donors and comparator groups of DBD donated lungs. Diamond *et al.*, reported in a large multicentre cohort study that the cause of donor death was not a risk factor for PGD[150].

Whilst perhaps lacking the exposure to some of the sequelae of brain-stem death, the lungs from the DCD donor are exposed to other potential injuries. In DCD donors, there may be longer periods of ventilation[145], an inevitable period of warm ischaemia[62] and the as-yet poorly characterised *agonal* phase which has some features of brain-stem death[151]. In the interval between discontinuation of support and procurement, organs are exposed to adverse conditions including hypoxia, hypotension and ischemia. White et al demonstrated that both hypoxic pulmonary vasoconstriction and a profound catecholamine surge occur following withdrawal of life-sustaining therapy (WLST) in a porcine model of DCD[152].

It is thus postulated that DBD donor lungs express higher levels of acute inflammation than lungs from DCD donors, and this offsets any injurious events around the donor's death and the delayed retrieval. However, with the exception of a single study by Kang *et al.*, analysing the differential gene expression profiles of DBD and DCD donor lungs[139]; objective supporting evidence has so far been lacking.

Ex-Vivo Lung Perfusion (EVLP) has emerged as a technology to evaluate and recondition lung grafts prior to transplantation[85] and now accounts for up to 20% of transplants in some centres with established EVLP programs[27, 153-155]. It has been argued that it may have specific benefits for lungs from DCD donors[156]. Nevertheless, it does allow better characterisation, both in terms of physiological measures and potential biomarkers, of both DCD and DBD lungs

Our group has previously demonstrated the feasibility of identifying pro-inflammatory signals in perfusate during EVLP of unsuitable donor lungs[83]. In this study, our objective was to establish if there are any differences in the pro-inflammatory profiles and expression of tissue injury markers between lungs from DBD and DCD donors before and during EVLP. We hypothesised that DBD lungs and their associated systemic inflammatory response following brain stem death would demonstrate increased levels of pro-inflammatory and injury markers when compared to DCD lungs from the outset of EVLP. Ischaemia reperfusion injury (IRI) and the associated excess risk of PGD arise not only from immunologic factors but also non-immunologic factors

related to the activation of a cascade of molecular events: activation of endothelial cells with increased expression of adhesion molecules increased adherence of leucocytes to the endothelium, and activation of the coagulation and complement systems. We evaluated a panel of inflammatory mediators, tissue injury-associated proteins and angiogenic factors in perfusate, bronchoalveolar lavage fluid (BALF) and lung tissue from human donor lungs undergoing clinical EVLP with intent for transplant. The panel was based on our group's previous work and available studies of biological markers in standard lung transplant and preclinical and clinical observations during EVLP[44, 83, 157, 158]. Our panel focussed on four main groups of markers to investigate specific pathways and processed involved; inflammation, tissue injury, endothelial barrier integrity and vascular remodelling.

Methods

Study subjects and Ex-vivo lung perfusion (EVLP) protocol

This was a retrospective study, performed using samples collected from a large cohort of clinical EVLP procedures performed as part of the DEVELOP-UK trial[98]. DEVELOP-UK was a multi-centre study including all 5 UK lung transplant centres. Fifty-three donor lungs, deemed unsuitable for immediate transplantation underwent EVLP. A standard lung procurement procedure was followed for donor lungs used for EVLP in the study[98]. EVLP assessments were performed using a Vivoline LS1 EVLP circuit (Vivoline Medical AB, Lund, Sweden) following 1 of 2 standardized perfusion protocols[98]. A standardised approach to obtaining BALF, perfusate and tissue specimens was followed in all cases.

Measurement of Inflammatory and Tissue Injury Markers in Bronchoalveolar Lavage Fluid (BALF), Perfusate and Tissue

BALF was collected by performing a flexible bronchoscopy in a sub-segmental bronchus of the right or left lower lobes. Sterile Saline 0.9% (120 ml) was instilled via bronchoscopy and suctioned back at the beginning of EVLP *before* ventilation was started. The procedure was repeated in the same lobe but different sub segmental bronchus at the end of perfusion.

Samples of perfusate were collected at longitudinal time-points throughout the EVLP. A control sample was collected from the primed EVLP circuit before starting donor lung perfusion. Repeated perfusate samples (5 ml) were then collected at 15 and 30 minutes after starting perfusion and every 30 minutes thereafter. The perfusate samples were centrifuged at 180 xg for 6 minutes at 4^{o}C to remove cellular debris. Supernatants were stored at -80^{o}C. Because priming volume was uniform, all protein expressions measured in perfusate were adjusted to the predicted total lung capacity (pTLC) of the donor as an estimate of perfused donor lung tissue volume and were reported as corrected perfusate concentrations (pg/ml). The pTLC was calculated using predicted values based on donor gender and height.

Tissue biopsies were taken from either the right middle lobe or lingula before EVLP was started using a stapler. These specimens were snap frozen in liquid nitrogen for protein analysis. For protein analysis snap frozen lung tissue (100 mg/sample) was homogenized in 500 µL homogenization buffer using a Minilys® ceramic bead tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Each homogenate was centrifuged twice at 13,000 xg for 5 minutes, the supernatants

decanted, and the protein concentration of each sample estimated using a Pierce™ BCA protein assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) before storage at -80ºC. 4 mg protein/ml diluted in 0.1% BSA in homogenization buffer was loaded onto protein assay plates.

The concentrations of a panel of pro-inflammatory cytokines and tissue injury markers were measured. Lactate dehydrogenase (LDH) levels were measured in perfusate, BAL and tissue lysate as per manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL). Perfusate and tissue lysates were analysed with a V-PLEX Human Biomarker 40-Plex Kit and a Human MMP 3-Plex Ultra-Sensitive Kit. BAL was analysed using a V-PLEX human pro-inflammatory Panel 1 Kit and a Human MMP 3-Plex Ultra-Sensitive Kit (Meso Scale Diagnostics, Rockville, MD). Human Syndecan-1 was measured using a sandwich ELISA (R&D Systems, Inc., Minneapolis, MN).

Statistical analysis

All statistics were performed using the GraphPad Prism 6 software package (GraphPad Software Inc., La Jolla, CA). Cytokine levels in BALF and tissue were compared between DBD and DCD lungs using Mann-Whitney U tests. A p-value of <0.05 was deemed statistically significant. Protein expressions in perfusate at each time-point were compared between DBD and DCD donor lungs with multiple t-tests. For comparisons in donor characteristics, the chi-square test or Fisher exact test were used for categorical variables and Mann-Whitney U tests for continuous variables.

Results

Study group and donor characteristics

Between April 2012 and July 2014, 53 donor lungs deemed unsuitable for immediate transplantation by all UK lung transplant centres were evaluated with EVLP within the DEVELOP-UK study. Seven donor lungs with incomplete perfusate sampling were excluded, leaving 46 donor lungs (35 DBD and 11 DCD) for this study. No significant differences between groups were observed for any of age, gender, or the duration of ventilation, **Table 4.1**.

Donor Variable	DBD (n=35)	DCD (n=11)	p-value
Age (yrs.)	48 (16-65)	53 (20-61)	0.1683
Female (%)	48.6	27.3	0.3024
Smoker (%)	48.6	45.5	>0.999
Optimised donor P/F (mmHg)	294	396	0.0906
Ischaemic time (min)	226	278	0.0255
Hybrid protocol	11	5	0.4766

Table 4.1 Demographic Characteristics of Donor Lungs.

P/F ratio: ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, Ischaemic time: duration from circulatory arrest in the donor to start of pulmonary artery (PA) perfusion on the EVLP circuit.

Pre-EVLP BALF samples were available from 40 donor lungs (31 DBD and 9 DCD) and pre-EVLP lung tissue biopsy samples from 33 donors. All available samples were included in this study.

Analysis of Bronchoalveolar lavage fluid, Perfusate and Tissue

Assessed lungs were divided into two groups based on donor type: DBD and DCD. Samples were analysed retrospectively for protein expressions. A total of 48 individual markers were detectable in perfusate.

Bronchoalveolar lavage fluid (BALF)

There were no significant differences observed for any of the inflammatory and injury marker levels investigated in BALF samples collected at the start of EVLP between DBD and DCD lungs, **Figure 4.1**.

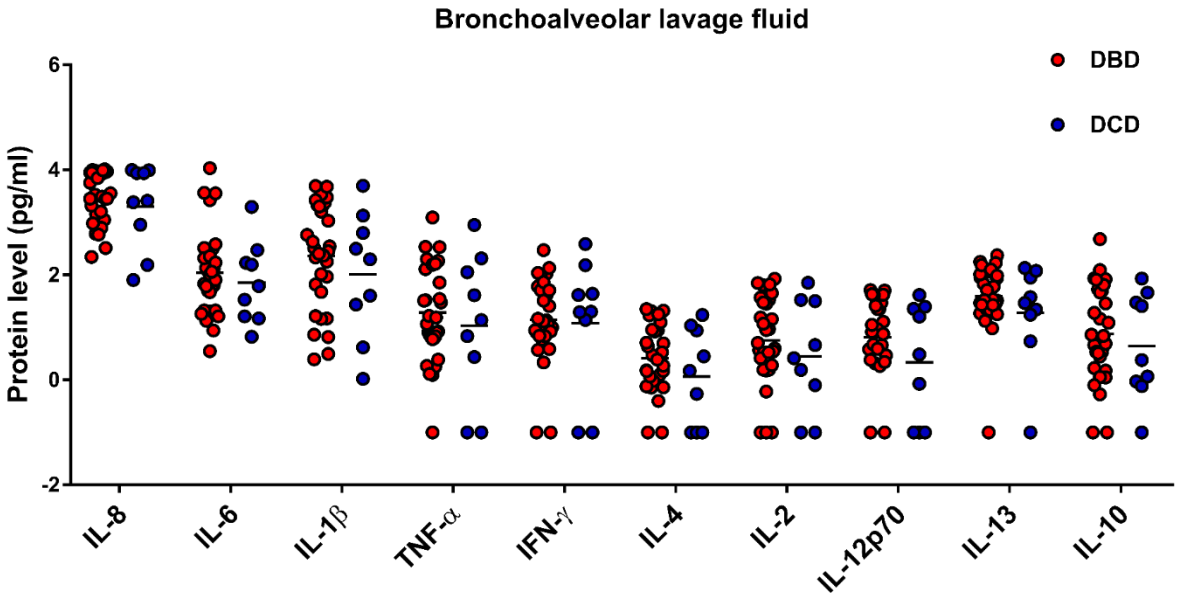

Figure 4.1 Inflammatory panel protein expression in BALF in DBD and DCD donor lungs prior to commencing EVLP

For all lungs, a research BAL was performed, instilling saline (40 ml) through the suction channel followed by gentle aspiration with sample collection prior to commencing ventilation at the beginning of EVLP. The BALF samples were filtered through a single layer of gauze to remove excess mucus and then centrifuged at 180 g for 6 minutes at 4 °C to separate the cellular component from the acellular supernatant. Supernatants were re-centrifuged at 700 g for 6 minutes at 4°C to remove any remaining cell debris, decanted and stored at -80 °C. Interleaved scatter plot with molecular marker levels expressed in (pg/ml) and black lines representing means. The protein expressions of a panel of pro-inflammatory cytokines detected using a V-PLEX human pro-inflammatory Panel 1 Kit and a Human MMP 3-Plex Ultra-Sensitive Kit (Meso Scale Diagnostics, Rockville, MD). DBD n=31, DCD n=9, analysed by Mann-Whitney U tests. There were no significant differences in the pro-inflammatory cytokine levels detected in BALF between DBD and DCD lungs. BALF: bronchoalveolar lavage fluid; EVLP: ex vivo lung perfusion; IL: interleukin; TNF-α: tumour necrosis factor alpha; IFN-γ: interferon gamma; DBD: donation after brain death; DCD: donation after circulatory death.

Tissue and Perfusate

There were discernible differences between DBD and DCD donor lungs detected in both lung tissue and perfusate samples taken during EVLP. We noted a pattern towards higher release of pro-inflammatory cytokines from tissue from DBD compared with DCD lungs. In lung tissue lysates, the levels of a large number of pro-inflammatory markers (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, IFN-γ, MCP-1, MCP-4, MCD, MIP-1α, TNF-α, TNF-β, Eotaxin and GM-CSF) were detected prior to EVLP commencing. A significant difference between the DBD and DCD groups was found for 4 of the proteins investigated. In 4 proteins measured in tissue, there was a significant difference between the groups, **Figure 4.2**. Tissue C-reactive protein (CRP) was found to be significantly higher in DBD lungs: median (DBD)=248,095.0 pg/ml, median (DCD)=151,905pg/ml, p=0.0077. Tissue IL-12p70 (p=0.028), IL-4 (p=0.0106) and IL-6 (p=0.0077) were also found in higher concentrations in DBD lungs.

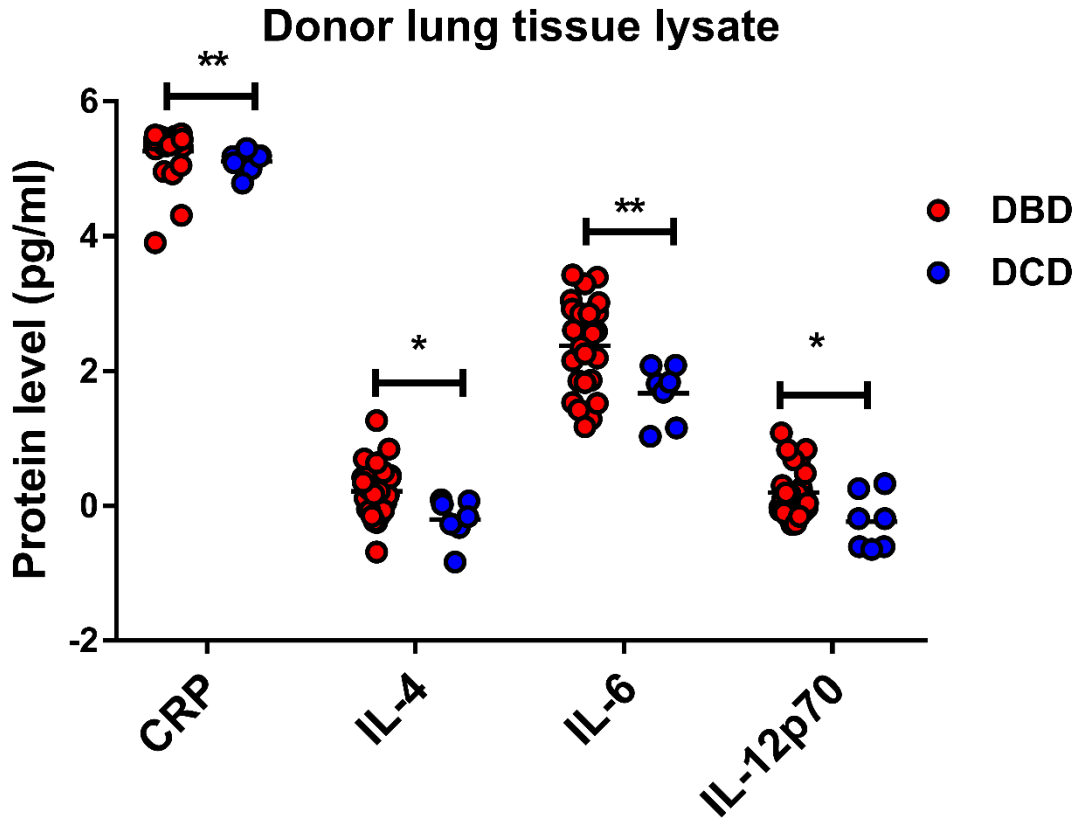

Figure 4.2 Inflammatory panel protein expression in lung tissue lysate in DBD and DCD donor lungs prior to commencing EVLP

*For all lungs, small biopsies of lung tissue (approximately 3x2x1 cm) were taken from either the right middle lobe or lingula just prior to the commencement of EVLP. The protein expressions of a panel of pro-inflammatory cytokines were detected with a V-PLEX Human Biomarker 40-Plex Kit and a Human MMP 3-Plex Ultra-Sensitive Kit as per manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL). Interleaved scatter plot with molecular marker levels expressed in (pg/ml) and black lines representing means. DBD n=26, DCD n=7, analysed by Mann-Whitney U tests, *P<0.05. **P<0.01 ***P<0.001. DBD lungs were found to express significantly higher levels of IL-4, IL-6, IL-12p70 and CRP in lung tissue. EVLP: ex vivo lung perfusion; CRP: c-reactive protein; IL: interleukin; DBD: donation after brain death; DCD: donation after circulatory death.*

Differences between DBD and DCD lungs were also seen in the concentrations of pro-inflammatory cytokines detected in perfusate. At 15 minutes into EVLP, there was a significant difference in Interleukin 1-beta (IL-1β) levels between DBD and DCD lungs: median (DBD)= 0.064 pg/ml (IQR 0.015- 1.54) and median (DCD)= 0.735 pg/ml (IQR 0.013-5.702), p=<0.01. At 30 minutes, a significantly higher level of Interleukin 1-alpha (IL-1α) was detected in the perfusate from DCD lungs: median (DBD)=0.031 pg/ml (IQR 0.014-1.738) and median (DCD)=1.078 (IQR 0.013-6.871), p=<0.01. This remained significant until the end of EVLP, **Figure 4.3.**

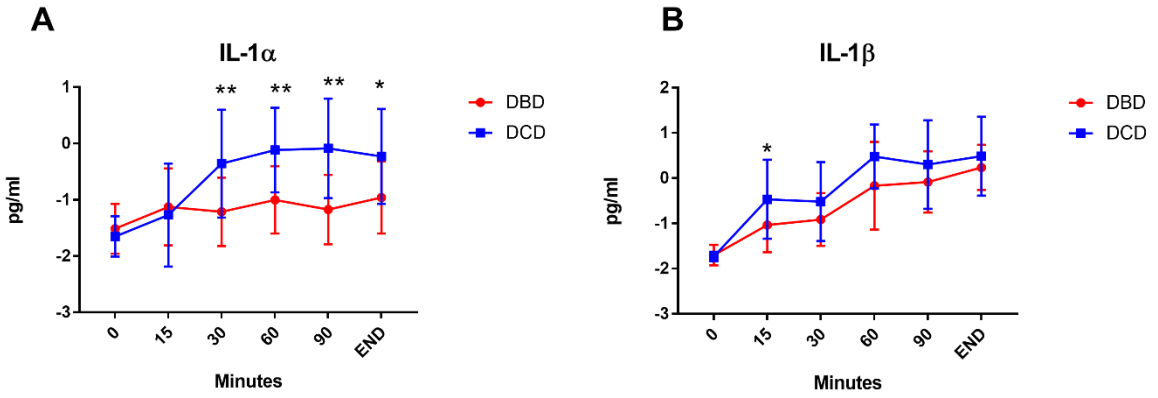

Figure 4.3 Perfusate protein expressions of IL-1 cytokines in DBD and DCD donor lungs during EVLP

*Repeated perfusate samples (5 ml) were collected before EVLP was started, after 15 minutes, at 30 minutes, every 30 minutes during perfusion and a final sample taken at the end of perfusion immediately before perfusion stopped. The protein expression reported as corrected perfusate concentrations (pg/ml) of IL-1α (Graph A) and IL-1β (Graph B) were detected with a V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics, Rockville, MD). Data are shown mean ± SD analysed using unpaired t-tests, DBD n=35, DCD n=11, *P<0.05. **P<0.01 ***P<0.001. Higher values of IL-1α and IL-1β was detected in DCD lungs compared with DBD lungs. A statistically significant difference was detected at the 15-minute time-point in IL-1β and from 30 minutes of EVLP in IL-1α. EVLP: ex vivo lung perfusion; IL: interleukin; DBD: donation after brain death; DCD: donation after circulatory death.*

The levels of Interleukin-10 (IL-10), a well-established anti-inflammatory cytokine were not significantly different between groups.

For an initial general assessment of tissue injury, we measured LDH levels, however no significant differences were seen between groups.

To assess the integrity of the pulmonary vascular compartment, we measured the endothelial barrier markers; soluble ICAM-1, VCAM-1 and Syndecan-1 levels as markers of endothelial glycocalyx disruption. There were no significant differences between the groups in these endothelial markers measured in tissue and perfusate **Figure 4.4 and Figure 4.5.**

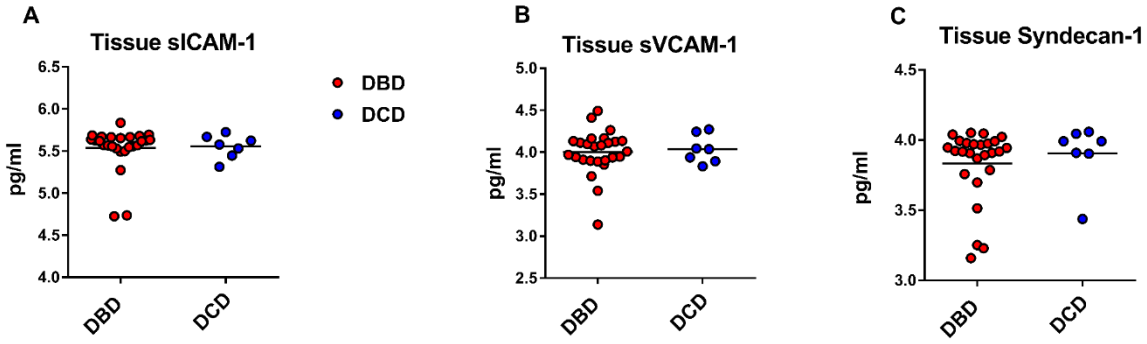

Figure 4.4 Protein expression of intracellular adhesion molecules in donor lung tissue lysates prior to commencing EVLP.

For all lungs, small biopsies of lung tissue (approximately 3x2x1 cm) were taken from either the right middle lobe or lingula just prior to the commencement of the EVLP process at the recipient hospital. The protein expressions of a panel of intracellular adhesion molecules were detected with a V-PLEX Human Biomarker 40-Plex Kit and a Human MMP 3-Plex Ultra-Sensitive Kit as per manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL). Interleaved scatter plot with molecular marker levels expressed in (pg/ml) and black lines representing means. DBD n=26, DCD n=7, analysed by Mann-Whitney U tests. Expression of soluble ICAM-1, VCAM-1 and Syndecan-1 in lung tissue lysate prior to commencing EVLP did not vary significantly between DBD and DCD lungs.

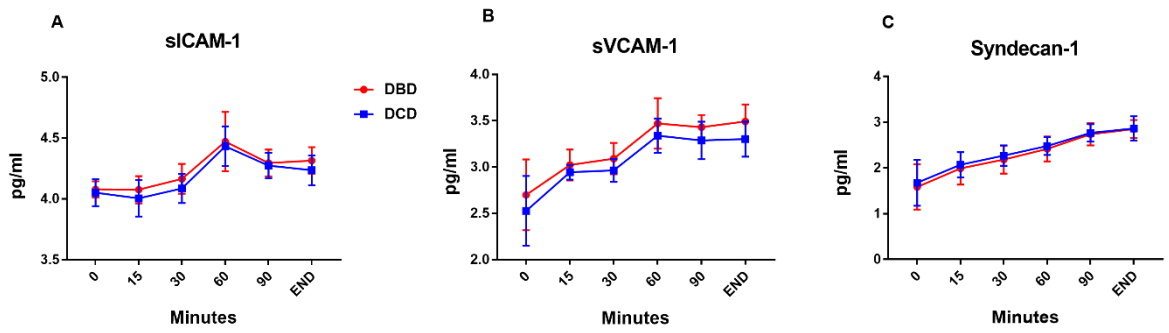

Figure 4.5 Protein expression of intracellular adhesion molecules in perfusate during EVLP

Repeated perfusate samples (5 ml) were collected from the perfusate sampling port before EVLP was started, after 15 minutes, at 30 minutes, every 30 minutes during perfusion and a final sample taken at the end of perfusion immediately before perfusion stopped. The protein expression of soluble ICAM-1(Graph A), VCAM-1(Graph B) and Syndecan-1 (Graph C) were detected with a V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics, Rockville, MD). Data are shown mean ± SD analysed using unpaired t-tests, DBD n=35, DCD n=11. Expression of soluble ICAM-1, VCAM-1 and Syndecan-1 during perfusion did not vary significantly between DBD and DCD lungs. EVLP: ex vivo lung perfusion; DBD: donation after brain death; DCD: donation after circulatory death.

We measured several angiogenic factors including placenta growth factor (PIGF) and vascular endothelial growth factor (VEGF) and found significantly higher levels in the perfusate of DBD lungs, **Figure 4.6**. At 15 minutes into EVLP, there was a significant difference in PIGF levels in perfusate between DBD and DCD lungs: median (DBD)= 1.640 pg/ml (IQR 1.002-3.090) and median (DCD)= 1.099 pg/ml (IQR 0.734-1.808), p=<0.01. The higher levels in DBD lungs remained significant throughout EVLP. The soluble portion of the fms-like tyrosine kinase (Flt-1) receptor (sFlt-1), an endogenous inhibitor of VEGF, thought to play an important role in endothelial dysfunction was also seen to be increased in DBD lungs. At 15 minutes into EVLP, there was a significant difference in sFlt-1 levels between DBD and DCD lungs: median (DBD)= 108.839 pg/ml (IQR 40.978-510.367) and median (DCD)= 25.290 pg/ml (IQR 15.440-120.719), p=<0.01. The higher levels in DBD lungs remained significant throughout EVLP.

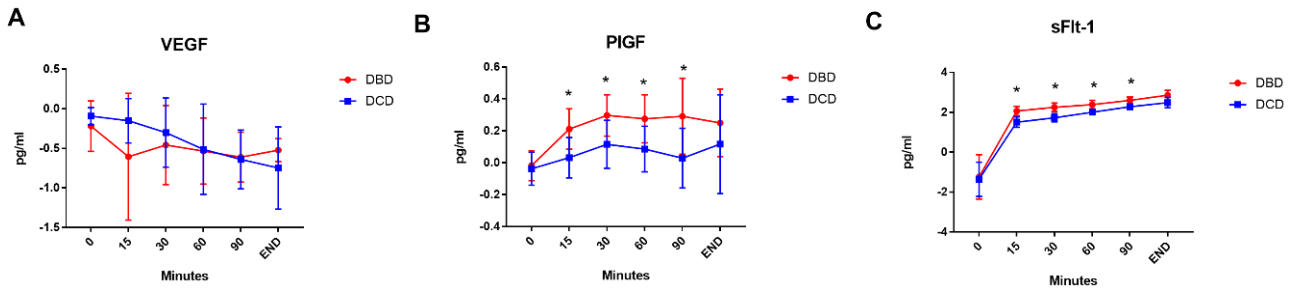

Figure 4.6 Perfusate protein expressions of angiogenic factors in DBD and DCD donor lungs during EVLP

*Repeated perfusate samples (5 ml) were collected from the perfusate sampling port before EVLP was started, after 15 minutes, at 30 minutes, every 30 minutes during perfusion and a final sample taken at the end of perfusion immediately before perfusion stopped. The perfusate samples were centrifuged at 180 g for 6 minutes at 4 °C to remove cellular debris. Supernatants were stored at -80 °C. All protein expressions measured in perfusate were adjusted to the donor predicted total lung capacity (pTLC), as an estimate of perfused donor lung volume, and reported as corrected perfusate concentrations (pg/ml). The protein expression of IL-1α (Graph A) and IL-1β (Graph B) were detected with a V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics, Rockville, MD). Data are shown mean ± SD analysed using unpaired t-tests, DBD n=35, DCD n=11, *P<0.05. **P<0.01 ***P<0.001. In DBD lungs, there are significantly higher levels of PIGF and sFlt-1 throughout perfusion. EVLP: ex vivo lung perfusion; VEGF: vascular endothelial growth factor; PIGF: placental growth factor; sFlt-1: soluble Fms-like tyrosine kinase receptor 1; DBD: donation after brain death; DCD: donation after circulatory death.*

Discussion

The increasing demand for donor lungs combined with largely static organ donation rates has led to the relaxation of donor inclusion criteria and acceptance of more extended criteria donor lungs for transplantation. One area of opportunity to increase the donor lung pool is the use of lungs from DCD donors, which have a prolonged period of warm ischaemia and go through an agonal phase but have traditionally been thought of as not being exposed to the systemic inflammatory stresses of brain stem death.

Our study aimed to investigate differences in the inflammatory profiles in the airway, vascular and tissue compartments between DBD and DCD lungs prior to and during EVLP. Our results demonstrate a difference in the pattern of pro-inflammatory protein and injury marker expression in tissue and perfusate samples from lungs that were from a DCD compared to a DBD donor.

Marker	DBD	DCD
Pro-inflammatory and tissue injury	Higher levels of IL-6, IL-4, IL-12p70, CRP	Higher acute phase cytokines: IL-1α and IL-1β
Anti-inflammatory	No differences	
Endothelial barrier markers	No differences	
Angiogenic markers	Higher levels of PIGF and sFlt-1	

Table 4.2 Summary table of the differences between DBD and DCD donor lungs during this study

BALF samples taken at the start of EVLP did not show any differences. BAL appears to show a weaker signal than that seen in perfusate and tissue. The reasons for this are unclear; however, one has to postulate that BAL sampling is more user dependent than perfusate sampling or tissue biopsying and whether this effects variability within a multi-centre cohort.

We have demonstrated that in extended criteria donor lungs which have been turned down for immediate transplantation, there are differential inflammatory and tissue injury marker profiles between DBD and DCD lungs. We found that IL-4, IL-6 and IL-12 were significantly higher in tissue samples from DBD lungs but this was not reflected

in the perfusate concentrations. We found that IL-α and IL-1β were detected in significantly higher levels in the perfusate of DCD lungs at as early as 15 minutes and throughout reperfusion. These observations demonstrate differences in the cytokine expression between DBD and DCD lungs and also in the compartment from which they are released.

It is generally accepted that IL-1β and TNF-α are released in the *early* post-injury phase, following acute brain injury[159]. Avlonitis *et al*. investigated the pathophysiology surrounding the events following brain stem death and the correlation with systemic inflammation. They demonstrated that early donor haemodynamic injury programs the donor lung to develop increased inflammation after reperfusion. They showed that it is the sympathetic discharge, so-called “catecholamine storm” and the hypertensive crisis following brain death that can cause neurogenic pulmonary oedema and reperfusion injury. They also showed that this effect could be prevented by donor pre-treatment with phentolamine, by preventing vasoconstriction and hypertension[160]. A subsequent study by the same group demonstrated that phentolamine pre-treatment prevented an increase in BALF cytokine levels. They suggested that the inflammatory lung injury observed after brain death is caused by haemodynamic stress produced by the catecholamine storm[161].

The release of acute phase cytokines is thought to result in the increased synthesis of IL-6, in addition to other mediators. IL-6 may act to decrease the production of IL-1β and TNF-α via a negative feedback mechanism[162]. It may be that in the DBD lungs, we missed an early peak of production of IL-1α and IL-1β and by the point of organ retrieval, this had diminished. Instead, we have seen the rise in mediators, IL-4, IL-6 and IL-12 following the stimulation of the “early response cytokines” IL-1 and TNF-α. Whereas due to the lung insults such as warm ischaemia occurring much closer to the time of retrieval in DCD lungs higher levels of the early response cytokines were elevated. This suggests that events surrounding death upregulate the early response inflammation in DCD donors. Whether the warm-ischaemic insults in DCD donors directly contributes to the increased IL-1β observed during EVLP requires further evaluation.

We have previously shown a relationship between perfusate IL-1β levels and subsequent lung allograft function after EVLP. Perfusate levels of IL-1β measured 30

min into EVLP could distinguish subsequent in-hospital mortality with a sensitivity and specificity of 100% and 1-year survival with similar precision[158]. The results here are in keeping with that finding, with perhaps DCD lungs being in a worse position. This flies against the accepted wisdom that DCD lungs are in every way equivalent to DBD lungs. As has been described, there is just one study suggesting a worse outcome in this cohort[148]

Hamill *et al*. reported that in 33 patients with traumatic brain injury, the levels of circulating catecholamines appeared to be excellent endogenous and readily quantifiable markers that reflected the extent of brain injury and predicted the likelihood of patient recovery[163]. Studies have also shown that there are differences in the catecholamine surge between sudden and gradual increases in intracranial pressure, with a sudden rise in ICP demonstrating a hyperdynamic state of greater degree and this coincides with a huge peak of catecholamines[164].

Catecholamine levels have also been shown to be important in the pathophysiology of DCD as well as in DBD donors. Ali *et al.* reported findings of a substantially more profound rise in catecholamines within the reperfused DCD donor compared to the DBD donor[165]. In the DCD donor, plasma epinephrine and norepinephrine concentrations began to increase after discontinuation of mechanical ventilation and continued to increase throughout circulatory arrest. At the end of the 15-min ischaemic period, the plasma norepinephrine concentration was 50-fold greater in the DCD donor than the peak concentration noted during brain stem death. Correspondingly the plasma epinephrine concentration was 30-fold greater than the peak value measured in the brain-dead donor. Catecholamine levels continued to increase during the first 5 min of extracorporeal perfusion and then began to decline. After 30 min of reperfusion, the norepinephrine and epinephrine concentrations in the DCD donor remained markedly elevated compared to baseline and were 2.5-fold and 2-fold greater, respectively than the peak levels seen after brain stem death.

Cytokine modulation during IRI and its correlation with donor parameters and graft function have been systematically explored in recent studies[166]. Machuca *et al.* studied the impact of EVLP on cytokines, chemokines, and growth factors and their correlation with graft performance; either during EVLP or post transplantation. They found that the best marker to differentiate declined lungs from control lungs was stem cell growth factor-β [$P < 0.001$, AUC (area under the curve) = 0.86] at 1 hour. The best

markers to differentiate PGD3 cases from controls were interleukin-8 ($P < 0.001$, AUC = 0.93) and growth-regulated oncogene-α ($P = 0.001$, AUC = 0.89) at 4 hours of EVLP[157].

Previous reports have demonstrated the detrimental effects of brain death on organs and the multifactorial pathophysiology, including haemodynamic, metabolic and inflammatory responses[37, 161, 167]. Several research groups have indicated that IL-6[37, 39] and IL-8[161] are amongst the most elevated cytokines in brain death.

IL-8, proposed as one of the optimum markers of EVLP transplant outcome in their study, has consistently shown potential in previous studies of donor lung injury from our group[43, 168]. De Perrot *et al*. also showed the importance of IL-8 in predicting early graft function[42]. In our study, there were no significant differences seen in the IL-8 expressions in perfusate and tissue between DBD and DCD lungs.

Our study showed significant differences in the expression of angiogenic factors between DBD and DCD lungs. We observed significantly higher levels of Placental growth factor (PlGF) and soluble Fms-like tyrosine kinase receptor 1 (sFlt-1) expression in the perfusate of DBD compared with DCD lungs. Placental growth factor (PlGF) is a member of the VEGF family of angiogenic proteins and is expressed in placental, cardiac, and lung tissue[169, 170]. PlGF is expressed in numerous cell types including endothelial cells, monocytes, and renal mesangial cells and activates sFlt-1. sFlt-1 is a member the vascular endothelial growth factor (VEGF) family and is a splice variant of the VEGFR-1 (Flt-1) receptor, which acts as an antagonist of VEGF and placental growth factor and is thought to be an antiangiogenic factor[171]. The pathogenic roles of sFlt-1 have been previously documented in preeclampsia [172, 173]and in chronic[174]and acute[175] renal disease. Chapal and co-workers reported that sFlt-1 is an independent risk factor for delayed graft function and to an early loss of peritubular capillaries during renal transplantation-related ischemia-reperfusion. They concluded that an increase in the potent antiangiogenic factor sFlt-1, combined with the repression of the proangiogenic VEGF, tips the balance toward a deleterious antiangiogenic state[176].

In patients with chest pain and acute coronary syndromes, higher PlGF levels are seen in those with myocardial infarction (MI) and are associated with an increased risk of short and long-term adverse outcomes[170, 177, 178]. Studies of circulating

sFlt-1 have demonstrated conflicting results, with some studies noting higher levels during acute MI compared to control patients [179] and others noting lower plasma levels in patients during the acute phase of MI compared to controls[180].

Ky *et al.* measured plasma PlGF and sFlt-1 in 1,403 patients from the Penn Heart Failure Study, a multi-centre cohort of chronic systolic heart failure (HF)[181]. Subjects were followed for death, cardiac transplantation, or ventricular assist device placement over a median follow-up of 2 years. They demonstrated that sFlt-1 was independently associated with measures of HF severity, including NYHA Class ($p<0.01$) and BNP ($p<0.01$).

One could postulate that the higher expression of angiogenic factors in DBD compared with DCD lung perfusate is a matter of the timing of the injury. Given that in DBD donors, the pathogenic process of brainstem injury has started hours if not days prior to organ retrieval, there has been time for the process of vascular remodelling endothelial dysfunction to begin. This is perhaps seen much later in DCD lungs and could perhaps be detected in the days following transplantation. A study measuring the levels of both PGIF and sFlt-1 in lung transplant recipients would help evaluate this further.

Literature comparing the differences between inflammation in DBD and DCD donor lungs at a cellular and molecular level is limited. Kang *et al*.[139] studied the differential gene expression profiles in lungs of DCD and DBD patients and interpreted the differences using functional pathway analysis. Analysis of gene expression profiles showed distinct activation of gene pathways between pre-transplant DBD and DCD human lungs. They found that there were 12 gene sets highly enriched in DBD but not DCD lungs. The gene sets were related to pathways of innate immunity (n=3), cytokine interaction (n=3), intracellular signalling (n=2), cell communication (n=2), apoptosis (n=1) and others (n=1). Increased expression of genes such as CXCL2, NAMPT, THBS1 and MMP9 imply activation of inflammatory, tissue injury pathways and cell communication in the DBD recovered organ. They demonstrated that lungs obtained from DCD donors had a significantly attenuated inflammatory transcriptional response compared with DBD donors, concluding that this suggests safe application of lung transplantation in properly preserved DCD donor lungs. In pre-transplant samples, cytokine levels were higher in DBD than for DCD. They demonstrated statistically significantly higher levels of IL-6 ($p=0.001$) in DBD compared with DCD lungs, which we have re-confirmed in this study.

Comparison studies have been carried out in the liver transplant community, as Xu *et al.* demonstrated significantly higher levels of neutrophil, T cell infiltration, and ICAM-1 expression detected in DBD compared to DCD donor liver prior to transplantation[182]. Their results demonstrated increased platelet adhesion and von Willebrand Factor expression prior to reperfusion in DBD livers. They concluded that as compared to DBD allografts, DCD grafts appear to have reduced leukocyte infiltration before and after transplantation. They suggested that DCD livers are prone to necrosis rather than inflammation.

Limitations of the Study

We have shown that in lungs submitted for EVLP (and therefore turned down for immediate transplantation) there was across a spectrum of inflammatory markers, specific differences in the inflammatory and tissue injury profiles comparing DBD and DCD lungs. A potential weakness is that these lungs come from one end of the spectrum of acceptability. Truly marginal donor lungs subjected to EVLP for reconditioning purposes form by nature a subpopulation that is likely to have a higher inflammatory burden than standard donor lungs. It may be that what might be termed “standard” as opposed to extended donors might have different inflammatory profiles.

Another limitation of our study is its relatively small sample size, with 46 human EVLP assessments included; however this cohort has previously been sufficient to demonstrate the feasibility of using perfusate markers to classify successful EVLP[83]. The results of this study need further evaluation in a larger prospective validation cohort of lungs exposed to ex vivo perfusion and that further investigation into the molecular and cellular differences of DBD and DCD lungs undergoing EVLP is welcomed.

In this study, we demonstrated in lungs subjected to EVLP (and therefore turned down for immediate transplant) that across a spectrum of markers, there are specific differences in the inflammatory and tissue injury profiles comparing DBD and DCD lungs. This study appears to be the first in-depth analysis of these inflammatory and injury markers in comparable groups of DCD and DBD donors and provides insight into the potential molecular events in the period prior to retrieval and differences between the two donor groups. Improved knowledge on when and how fast brain death related processes occur and the sequence in which these events take place, will help us to understand the primary triggers of DBD-associated organ injury. A greater

understanding of pre-donation events may inform the creation of donor management protocols tailored specifically to the DCD and DBD lung and evaluation strategies that identify organs suitable for transplantation. Further knowledge of the pathophysiological processes occurring in these groups may identify ways to improve donor management and possible therapeutic targets for improving outcomes in lung transplantation.

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CHAPTER 5

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EVLP as a platform for novel therapeutics: the effect of sildenafil

Chapter 5: EVLP as a platform for novel therapeutics: Sildenafil

In the previous chapters, I have reported on *Ex-vivo* lung perfusion (EVLP) emerging over the past decade as a proficient technique to objectively assess and recondition organs unsuitable for immediate transplantation. In addition to its function as a donor lung assessment platform, the EVLP technique offers an opportunity to attenuate the inflammatory response and encourages the recovery of vascular integrity in the donor lung before implantation, with a personalised medicine approach. Better knowledge of the biology of these lungs and ways to favour cellular recovery may improve early outcomes after lung transplantation and further help to safely maximise lung use from the existing donor pool.

As part of the BTRU, my prospective work concentrated on developing a robust, reproducible model for EVLP of donor lungs that have been turned down for immediate transplantation and evaluating this as a platform for therapeutic interventions. As described in Chapter 2, training and education in the porcine model was utilised to develop, optimise and establish a research EVLP model for human lungs turned down for transplantation and use as a platform for delivering a novel therapeutic.

Armed with the knowledge from the literature review presented in Chapter 1, on primary graft dysfunction and ischaemia reperfusion injury, I wanted to investigate a therapy which had been presented in the literature to have potential attenuating effects of this pathological process. In addition, we set out to demonstrate the feasibility and limitations of using EVLP as a platform for therapy delivery.

The therapeutic agent, sildenafil citrate, a selective and potent inhibitor of phosphodiesterase type 5 (PDE-5) targets the nitric oxide (NO)/ cyclic guanosine monophosphate (cGMP) pathway in the vascular endothelium. It is a vasodilator commonly used in pulmonary hypertension and has the potential to improve the physiology of donor lungs by reducing pulmonary vascular resistance and pulmonary artery pressure. It has also been postulated to have an immunomodulatory effect, via NO inhibition of adhesion molecule expression, leucocyte adhesion and platelet aggregation. In an effort to clarify the haemodynamic response to intravenously administered sildenafil citrate and investigate its potential use in the EVLP setting, we conducted a prospective study of 5 human donor lungs that had been turned down for transplantation. We aimed to study the effects of sildenafil on lung physiology during EVLP, in addition to its biological effect on cytokines, endothelial markers and angiogenic factors. The following chapter outlines this study. I was responsible for

the study concept, design and execution. Laboratory analyses were performed by myself, with the introduction to the laboratory techniques generously provided by members of the Borthwick Fibrosis Research Group. I led the subsequent data analysis and wrote the manuscript for the study which is planned for submission for publication.

Introduction

Despite 20 years of successful lung transplantation, primary graft dysfunction (PGD) remains a significant and devastating complication in the early postoperative lung transplant period[183, 184]. PGD, a form of acute lung injury (ALI) occurring within 72 hours of lung transplantation, is characterised by hypoxaemia and alveolar infiltrates in the allograft[95]. Occurring in up to 20% of lung transplant recipients, PGD results in the development of progressive hypoxaemia, increased pulmonary pressures, and pulmonary oedema[185]. Patients in whom PGD develops have increased rates of graft rejection, infection, longer intensive care unit and hospital lengths of stay, and greater short and long-term mortality[186-188].

Alterations in pulmonary vascular resistance, microvascular permeability, and gas exchange support the belief that the injury in PGD is primarily caused by IRI[189].IRI refers to sterile inflammation that occurs after substrate supply is restored following a period of absent blood flow[190]. A long ischaemic period followed by quick restoration of blood and oxygen supply to the lung can lead to IRI that is characterised by damage to the lung structure and a decline in respiratory function. Lung IRI is characterised by massive pulmonary oedema and hypoxaemia, which are difficult to reverse and require intensive ventilator therapy. The alveolar air spaces which are the site of gaseous exchange are separated from the circulating blood volume only by the alveolar-capillary membrane, a monolayer of endothelial cells fused via the extracellular matrix to the alveolar epithelial cells[191]. The pulmonary endothelium is the most vulnerable component of this structure[192]. Permeability occurs predominantly via a para-cellular route between two cells, normally “zipped together” by junctional proteins. Several strategies have also been introduced into clinical morbidity and mortality after lung transplantation. Several strategies have also been introduced into clinical practice for the prevention and treatment of ischaemia–reperfusion with various degrees of success.

Several studies have suggested an important role for NO signalling pathways mediated by secondary messengers such as cGMP in the maintenance of vascular homeostasis and have been implicated in IR lung injury. cGMP is an important signalling molecule that stimulates smooth muscle relaxation. Models of lung IRI have demonstrated that reduced levels of cGMP[193] after lung reperfusion have been associated with the development of pulmonary hypertension,[194] reduced oxygenation,[195] and increased microvascular permeability[189].

PDE-5 inhibitors such as sildenafil prevent the breakdown of NO driven cGMP[196]. Sildenafil is widely used in the treatment of pulmonary hypertension and erectile dysfunction[196, 197]. However, cGMP also has other regulatory roles that stimulate neutrophil degranulation, inhibit platelet aggregation, regulate intracellular calcium levels, and open the mitochondrial K+ATP channels[198, 199]. These mechanisms have a beneficial role in reducing the severity of IRI. Previous studies have reported that sildenafil can suppress multiple pro-inflammatory cytokines which play an important role in neutrophil and monocyte recruitment[200]. It has also been reported that through modulation of the NO/cGMP pathway, the interaction between neutrophils and endothelial cells via adhesion molecules can be attenuated[201].

The importance of NO signalling via cGMP suggests an opportunity for preconditioning via modulation of this pathway. Through augmentation of cGMP levels, PDE inhibition may protect against the deleterious effects of IRI after lung transplantation. Although there have been several animal studies investigating the use of phosphodiesterase inhibitors as a preconditioning treatment of IRI, there is a limited number of studies assessing the potential therapeutic effects in human donor lungs.

One of the major upcoming challenges will be to improve the number of donor lungs available for transplantation. Although the number of patients on the waiting list continues to increase, only 10 - 30% of the available donor lungs are clinically used. Hence, the development of new strategies to repair and improve the quality of the lungs could have a tremendous impact on the number of transplants performed. EVLP has emerged as a promising technique for assessing and potentially reconditioning unusable human donor lungs before use in clinical lung transplantation. More targeted interventions are now possible with the use of EVLP prior to transplantation. EVLP provides us with a highly unique, localised environment for the administration of high-dose treatments to donor lungs prior to implantation without fear of systemic repercussions. Given the potential therapeutic role for PDE inhibition in PGD, we hypothesised that administering sildenafil to human donor lungs during EVLP would reduce the potential for IRI and improve lung physiology during perfusion. The study aimed to examine the physiological, immunological and histological changes in donor lungs deemed unsuitable for standard transplantation undergoing EVLP with a perfusate supplemented with the known vasodilatory agent, Sildenafil.

Materials and Methods

Study design

This was a prospective observational study examining the effect of sildenafil administered to human donor lungs during EVLP. Ethical approval was received from the local Research Ethics Committee and informed consent obtained from donor families (REC 16/NE/0230). Inclusion and exclusion criteria are listed in **Table 5.1**

INCLUSION CRITERIA	EXCLUSION CRITERIA
• Patient identified as a potential organ donor by the NHS-BT • Age between 18-85 years • Must fall into one of the following groups: * defined brain death * individuals who have had a withdrawal of care and a minimum 5-minute observation period to confirm cardiac death. • Only patients with appropriate consent reflecting their own and their families' wishes will be considered. • To be eligible for recruitment into the study, the tissue in question will have been declined for clinical use by all transplant centres in the UK.	• Under the age of 18 or above age 85 • Presentation of blood borne pathogens such as HIV, Hepatitis B, Hepatitis C • Tuberculosis (TB) • Clostridium difficile (CD) • Methicillin resistant staphylococcus aureus (MRSA).

Table 5.1 Study inclusion exclusion criteria

Ex-vivo lung perfusion protocol

All lungs included in this study were adult donor lungs with donor-family research consent deemed unsuitable for lung transplantation by all UK lung transplant centres. Adult donor lungs rejected for standard lung transplantation and meeting the above criteria were procured according to established clinical protocols, inflated with 100% oxygen and stored in cold Perfadex^{TM} for transport to our institution. The standard lung procurement procedure can be briefly summarised as follows; lungs were anterogradely flushed with supplemented Perfadex® (XVIVO Perfusion AB, Gothenburg, Sweden) [3.3 ml of 3.6% trometamol (THAM), 0.6 ml of calcium chloride

(CaCl2) ± 2.5 ml of prostacyclin/l], initially at room temperature and then the remainder at 4°C. A minimum volume of 60 ml/kg was given. After the antegrade flush, 200 ml was flushed into each pulmonary vein as a final retrograde flush. The retrieved lungs were filled with air, the airway sealed and then sterilely bagged for transport on ice, during a period of cold storage. All EVLP procedures were carried out in the Transplant Regenerative Medicine Facility, NHS-BT Donor Centre, Holland Drive, Newcastle, NE2 4NQ.

EVLP assessments were performed using a Medtronic EVLP Rig. The technique for EVLP followed a protocol described in detail by the Toronto group[88], which included a perfusate consisting of 2 litres of acellular Steen solution with 10,000 units of heparin. The EVLP protocol is outlined in **Table 5.2.**

PERFUSION	
Target flow	40% of cardiac output
Pulmonary arterial pressure	<20 mmHg
Left atrial pressure	3-5 mm Hg (Closed LA)
Pump	Roller
Perfusate	2L Steen Solution™
VENTILATION	
Mode	Volume controlled
Tidal volume	7 ml/kg
Frequency	7 bpm
Peak end-expiratory pressure	5 cm H2O
Fraction of inspired oxygen	50 %
TEMPERATURE	
Start of ventilation	32ºC
Start of perfusion	15ºC
Start of evaluation	37ºC

Table 5.2 EVLP protocol used during this study

Sildenafil Citrate

In all lung pairs within the treatment group, the selective PDE-5 inhibitor sildenafil (Revatio; Pfizer, Sandwich, Kent) was administered at a dose of 10 mg, as a bolus into the arterial arm of the circuit after a period of stable normothermic perfusion, 30 minutes of ventilation and when the lungs had reached 37 °C.

Physiological Assessment

Haemodynamic data was continuously monitored throughout EVLP and recorded at 5 minute intervals prior to sildenafil delivery, at one minute intervals for the subsequent hour and then every 15 minutes until the end of perfusion. The aim was to compare the haemodynamic data before and after sildenafil was administered and identify any alteration to the physiological parameters. The pulmonary artery flow (L/min), i.e. cardiac output (CO) was measured continuously via a flow probe in the ex vivo model and the pulmonary artery pressure (PAP) recorded via a pressure transducer positioned in the PA. Temperature recordings were taken from the left atrium as to obtain readings of the perfusate leaving the lungs. Blood gas analyses using an ISTAT portable machine were carried out prior to commencing EVLP and at 30-minute intervals during perfusion.

Sample collection

Lung tissue

Small biopsies (approximately 3x3x1 cm) of lung tissue were taken using a Covidien Duet (absorbable buttressed) endo-GIA stapler from either the right middle lobe or lingula at four time points:

Biopsy 1: Taken prior to commencement of the EVLP process

Biopsy 2: 30 minutes' post drug dosing

Biopsy 3: 60 minutes' post drug dosing

Biopsy 4: Taken at the end of the EVLP process once perfusion has stopped

Biopsies were placed on sterile gauze dampened with 0.9 % sodium chloride in a sample pot and the pot stored on ice until processing. From each of these biopsies, a small amount snap frozen using dry ice and isopentane slurry for subsequent mechanistic studies. The remaining tissue was fixed in formalin, paraffin embedded and sections cut for Haematoxylin and Eosin staining for routine histological

evaluation. Tissue blocks were available for subsequent immune-localisation studies using immunohistochemistry.

Perfusate fluid

Repeated perfusate samples (5 ml) were collected from the perfusate sampling port:

- Perfusate 0: Perfusate primed EVLP circuit before the donor lung perfusion was started.
- Perfusate 1: 15 minutes after perfusion start.
- Perfusate 2: 30 minutes after perfusion start.
- Perfusate 3 to Perfusate 8: Every 30 minutes of perfusion.
- Perfusate X: End of perfusion immediately before perfusion stop.

The perfusate samples were centrifuged at 180 xg for 6 minutes at 4 °C to remove cellular debris. Supernatants were stored at -80 °C.

Quantification of protein expressions in perfusate and bronchoalveolar lavage fluid

All protein expressions measured in perfusate were adjusted to the donor predicted total lung capacity (pTLC), as an estimate of perfused donor lung volume, and reported as corrected perfusate concentrations (pg/ml). The pTLC was calculated in a routine fashion based on donor gender and height[202].

Meso scale discovery multiarray

To assess the effect of sildenafil on the concentrations of pro-inflammatory cytokines detected during perfusion an MSD Multi-Array® U-PLEX Human Biomarker pro-inflammatory Panel 1 Kit (K15067L-1) (Meso Scale Diagnostics, LLC, Rockville, MD) was used. This allowed for the detection of the specific cytokines of interest, IL-1β, IL-6, IL-8, TNF-α, and VEGF which have been highlighted in previous EVLP literature[43, 83, 157]. Perfusate samples were also analyzed with a R-PLEX Human Ang-2 Antibody Set (F21YR-3) (also Meso Scale Diagnostics, LLC, Rockville, MD). The assays were performed according to manufacturer's instructions. Technical issues prevented reading of perfusate samples from donor lung EVLP03 and these samples were therefore excluded from the analysis.

Enzyme-linked immunosorbent assays (ELISAs)

To assess the effect of sildenafil on the integrity of the pulmonary vascular compartment, we measured a number of endothelial barrier markers, including junctional proteins and thrombogenic agents which are known to activate the

pulmonary endothelium. Solid phase sandwich ELISAs were used to assess the perfusate concentration of five soluble markers of: E-selection (DY724), Von Willebrand factor (DY2764-05), Thrombomodulin (DY3947), VE-cadherin (DY938-05) (all R&D Systems, Inc., Minneapolis, MN). These were measured with commercially available ELISA kits according to manufacturer's instructions.

Griess Reagent System

To investigate nitric oxide formation, nitrite (NO_2^-) was measured, one of two primary, stable and non-volatile breakdown products of NO. The Griess Reagent System (G2930 from Promega) is based on the chemical reaction which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.

Histology

Lung biopsy tissue was taken from pre- and post-EVLP for all six human lungs and at two additional time points (30 minutes' post sildenafil and 60 minutes' post sildenafil) in H004, H005 and H006 (n=18 sections). Tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. 5 µm sections were cut and mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, USA). The sections are deparaffinised in xylene before rehydration through graded alcohol solutions. Endogenous peroxidase was blocked in methanol/hydrogen peroxide (1:50) and incubated for 30 minutes. Slides are washed in PBS between all major steps. Immunolocalization of neutrophil elastase (EPR7479) and e NOS (ab5589) (both Abcam, Inc., Cambridge, MA) was by the avidin-biotin method with biotinylated secondary antibodies and subsequent DAB Chromogen staining (Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with Mayer's haematoxylin, dehydrated, cover slipped, and digitally photographed. Five fields at x20 magnification from each section were independently scored by two blinded arbiters using a semi-quantitative scoring system with 4 levels: (0 = no staining; 1 = weak staining, 2 = moderate staining; and 3 = strong staining). Average scores were compared between groups. Two independent arbiters individually counted neutrophil elastase positive cells and average scores were recorded.

Statistical analysis

All data measurements are presented as the mean ± standard error of the mean. Comparison of the mean values was performed by using repeated measures analysis of variance (ANOVA method). Differences were considered significant when

the p-value was less than 0.05. Donor characteristics and physiological parameters are expressed as medians with interquartile range. Paired samples, start-end of perfusion, were compared with Wilcoxon signed-ranks tests. Log protein expressions were compared between transplanted and non-transplanted lungs with multiple t-tests.

Results

Study group

Lungs from six UK multi-organ donors were identified as unsuitable for immediate standard transplantation despite extensive donor management and were assessed with EVLP in the study. The donor demographics and indications for EVLP are shown in Table 3.

Donor characteristics

Six donor lungs, all bilateral were recruited into the study. The EVLP assessments were evenly distributed between genders, 3 females (50%) and 3 males (50%). The median donor age was 54 years (range 28-64 years). Donor lungs came from 5 heart beating donors (DBD) and 1 donation after circulatory death (DCD).

Of these six donors, two died from spontaneous intracranial haemorrhage (33.3%), two from hypoxic brain injury (33.3%), one from ischaemic stroke (16.6%) and one from meningitis (16.6%).

Of six donors, 4 (66.6%)were never smokers, 1 ex-smoker (16.6%)and 1 current smoker (16.6%). Three (50%) had an abnormal chest x-ray at the time of procurement, and 2 (33.3%) had airway secretions deemed prohibitive of standard transplantation; predominantly purulent secretions. The median ventilation time for the EVLP donors before procurement was 2 days (range 2 to 5 days) and 2 donors (33.3%) had positive microbiology cultures from either sputum, BAL or CSF. The median optimised PaO_2:FiO_2 for the 6 EVLP donors at the time of procurement on 100% inspired oxygen and PEEP of 5 cmH_2O was 56.5 kPa. Donor characteristics are listed in **Table 5.3.**

Variable	H001	H002	H003	H004	H005	H006	Median (range)
Sex	Female	Male	Male	Female	Male	Female	
Age (years)	56	28	42	56	52	64	54 (28-64)
Height (cm)	165	176	171	170	174	163	170.5 (163-176)
Weight (kg)	65	80	76.5	70	68	75	72.5 (70-80)
Blood group	A+	O+	A+	O-	A+	A+	
Cause of death	Hypoxic brain damage	Hypoxic brain damage	Intracranial CVA	Intracranial haemorrhage	Meningitis	Intracranial haemorrhage	
Donor type	DCD	DBD	DBD	DBD	DBD	DBD	
Smoking	Never	Smoker	Never	Never	Never	Ex-smoker	
Chest x-ray findings	Right pneumothorax.	Consolidation left base	Bi-basal consolidation	Clear	Clear	Clear	
Microbiology on offer	Nil	Nil	*Staphylococcus aureus* in sputum	Nil	*Neisseria meningitidis*	Nil	
Secretions	Nil	Yes	Yes	Nil	Nil	Nil	
Duration of ventilation (days)	2	3	5	2	4	1	2.5 (1-5)

Table 5.3 Donor Characteristics.

Variable	H001	H002	H003	H004	H005	H006	Median (range)
Ischaemic time (hours)	**28**	**21**	**26**	**14**	**14**	**17**	19 (14-28)
EVLP time (mins)	210	225	165	160	160	155	162.5 (155-210)
Optimised Donor P/F ratio (kPa)	63.8	29.9	50.7	55.6	57.5	64	56.55 (29.9-64)
P/F ratio after EVLP (kPa)	48.3	75.24	34.6	64.9	61.3	43.6	54.8 (34.6-75.24)
Perfusion flow (L/min)	3.71	1.9	1.85	1.69	2.0	1.92	1.91 (1.69-3.71)
Pulmonary artery pressure (mmHg)	15	17	17	5	7	8	11.5 (5-17)
Left atrial pressure (mmHg)	0	3	3	3	5	3	3 (0-5)
PVR (dyne · s/cm5)	827	507	1806	347	224	457	482 (224-1806)
Peak airway pressure End (cmH_2O)	19	13	17	13	18	14	15.5 (13-19)
Compliance	NA	NA	46.2	48	34.6	41	43.6 (41-48)

Table 5.4 Lung Reconditioning

Ex-vivo lung perfusion outcomes

After reconditioning, the lungs from these donors produced a median PaO2:FiO2 on a 100% oxygen of 54.8 kPa. The median CIT from donor lung flush to start of EVLP was 19 hours (range 14-28 hours) and median perfusion time was 162.5 min (range 155-225 min). The variables of the physiology achieved, hemodynamic and ventilatory parameters are presented in **Table 5.4.**

Effect of sildenafil on ex vivo lung physiology

Haemodynamic data including pulmonary artery flow (L/min), i.e. cardiac output (CO) in the ex vivo model and pulmonary artery pressure (PAP), were measured continuously. Pulmonary vascular resistance was calculated retrospectively, determined as follows:

$$PVR(\text{dynes - sec - cm - 5}) = 80 * (\text{mean pulmonary artery pressure - left atrial pressure})/\text{pulmonary flow}$$

In the five donor lungs that received Sildenafil, changes in mean PA pressure, mean aortic pressure, pulmonary flow and pulmonary vascular resistance did not change significantly following administration of the sildenafil bolus, **Figure 5.1**. By analysis of variance with repeated measures, there were no significant differences between any of the time points or comparing time points following administration of the drug to the baseline parameters.

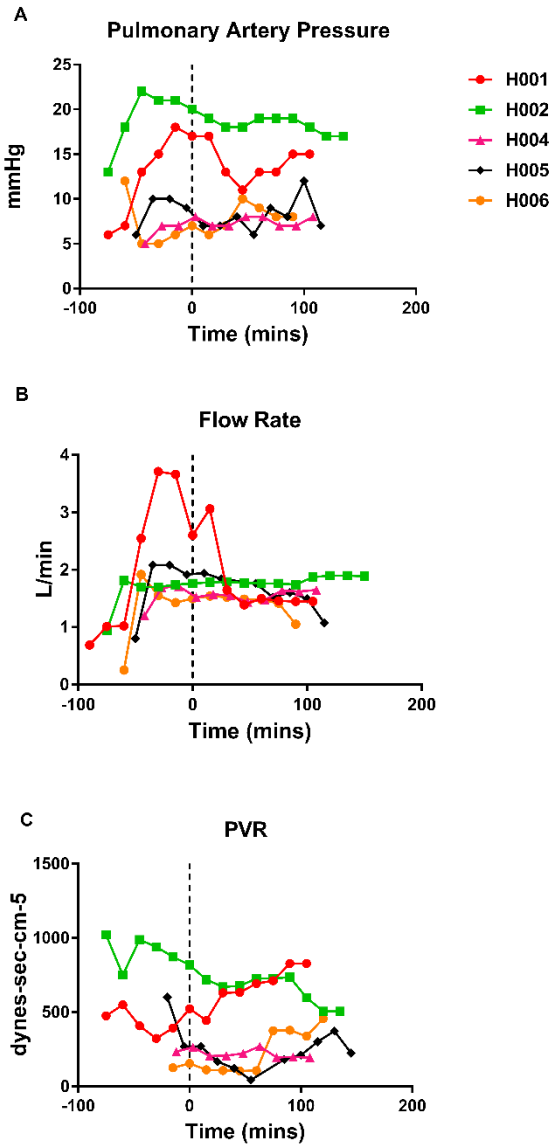

Figure 5.1 Effect of sildenafil on the physiological characteristics of EVLP donor lungs

Line graphs show the physiological data over the course of EVLP; pulmonary artery pressure (PAP) (graph A); flow rate (graph B) and pulmonary vascular resistance (PVR) (graph C). The dotted black line indicates the time at which 10 mg sildenafil was administered. Data analysed using repeated measures ANOVA, n=5.There were no significant changes in physiology when comparing between each time-point or when using time 0 as a comparison. In PAP, there was a downward trend following sildenafil in H001 and H002 where there were higher pressures recorded prior to Time 0. EVLP denotes ex-vivo lung perfusion, Time in minutes of ex vivo lung perfusion prior to and after sildenafil administration.

In all lungs, Pa02:Fi02 remained stable and above 40kPa (300mmHg) throughout EVLP. No significant changes were observed following sildenafil, **Figure 5.2.**

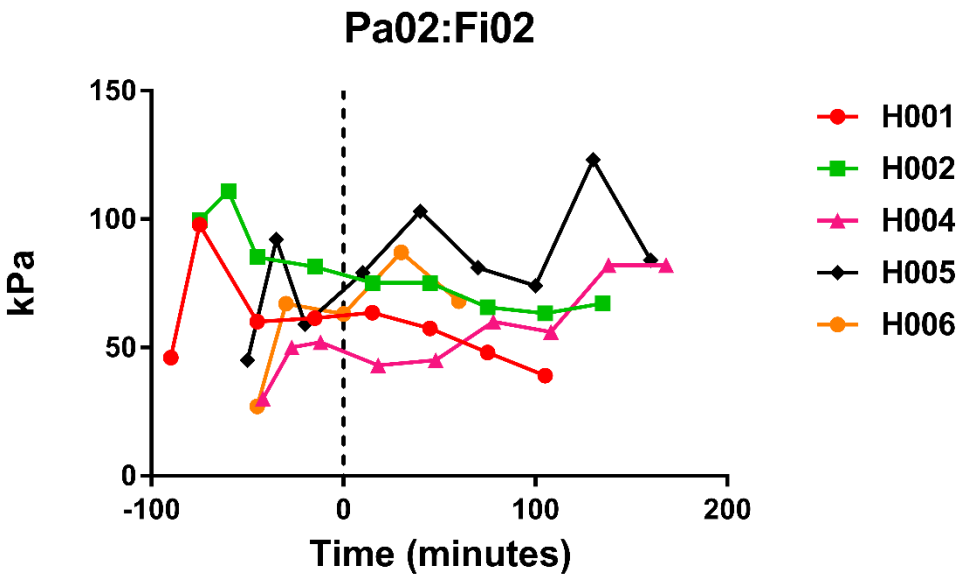

Figure 5.2 Effect of sildenafil on oxygenation during EVLP

In all lung pairs shown, the selective PDE-5 inhibitor sildenafil (Revatio; Pfizer, Sandwich, Kent) at a dose of 10 mg was administered as a bolus into the arterial arm of the EVLP circuit after a period of stable reperfusion, 30 minutes of ventilation and when the lungs had reached 37˚C. Line graphs show the PaO2/FiO2 ratio which denotes oxygenation capacity over the course of EVLP. The dotted black line indicates the time at which sildenafil was administered. Data analysed using repeated measures ANOVA, n=5.There were no significant changes in oxygenation when comparing between each time-point or when using time 0 as a comparison. EVLP denotes ex-vivo lung perfusion, Time in minutes of ex vivo lung perfusion prior to and after sildenafil administration. PaO2/FiO2 ratio denotes the ratio of arterial oxygen partial pressure to fractional inspired oxygen.

To investigate nitric oxide formation, NO_2^- was measured, a primary, stable and non-volatile breakdown product of NO. As expected following the delivery of sildenafil to the perfusate, an increase in nitrite concentration was seen. There was a significant increase in nitrite concentration when comparing concentrations at 45 minutes following sildenafil administration versus baseline levels, (p=0.0261). The nitrite concentrations are demonstrated in **Figure 5.3.**

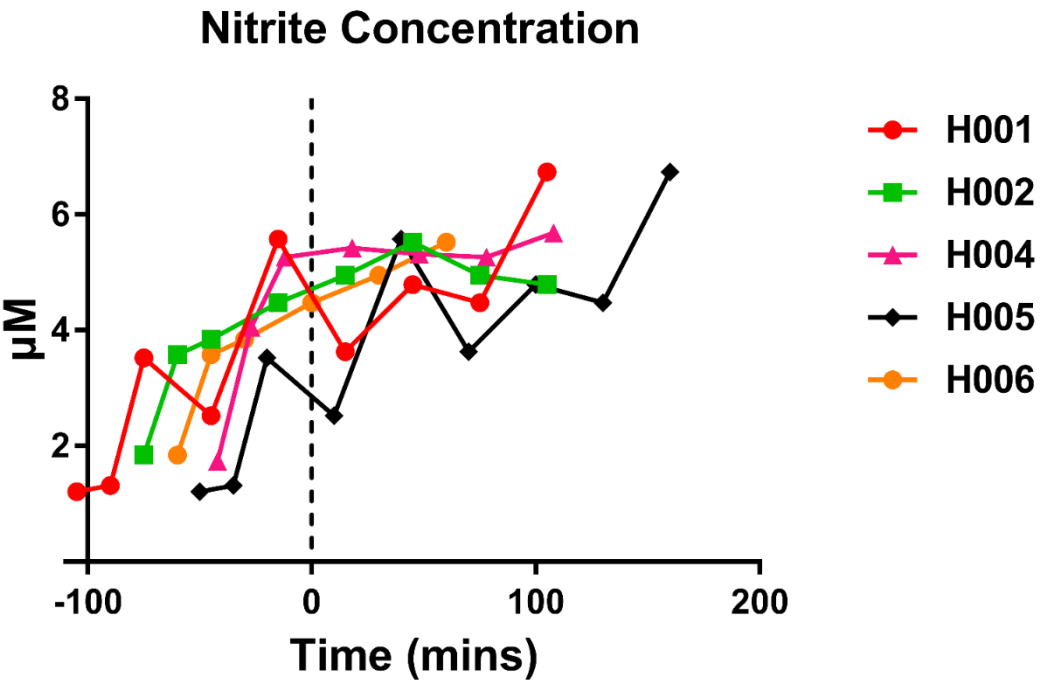

Figure 5.3 Effect of sildenafil on nitrite concentration in perfusate

In all lung pairs shown, the selective PDE-5 inhibitor sildenafil (Revatio; Pfizer, Sandwich, Kent) at a dose of 10 mg was administered as a bolus into the arterial arm of the EVLP circuit after a period of stable reperfusion, 30 minutes of ventilation and when the lungs had reached 37°C. The black dotted line indicates time 0, when sildenafil was given. Perfusate samples were collected from the circuit prior to commencing EVLP, at 15 minutes and then at 30 minute intervals during perfusion. The Griess Reagent System (G2930 from Promega) was used to detect nitrite concentration in each sample. By analysis of variance with repeated measures, a significant difference was seen at 45 minutes after sildenafil was given versus baseline values, P=0.0261. EVLP denotes ex-vivo lung perfusion, Time in minutes of ex vivo lung perfusion prior to and after sildenafil administration.

Effect of sildenafil on cytokine expression

When comparing the concentrations of cytokines IL-1β, IL-6, IL-8 and TNF-α in the perfusate of the 5 donor lungs that received sildenafil versus the historical DEVELOP-UK cohort[98] significant differences were seen at the final sample time, with higher concentrations of TNF- α and IL-8 in the sildenafil group. The overall trends for these pro-inflammatory cytokines were very similar in both groups, **Figure 5.4**. The effect of sildenafil on cytokine protein levels can be seen in **Figure 5.5.**

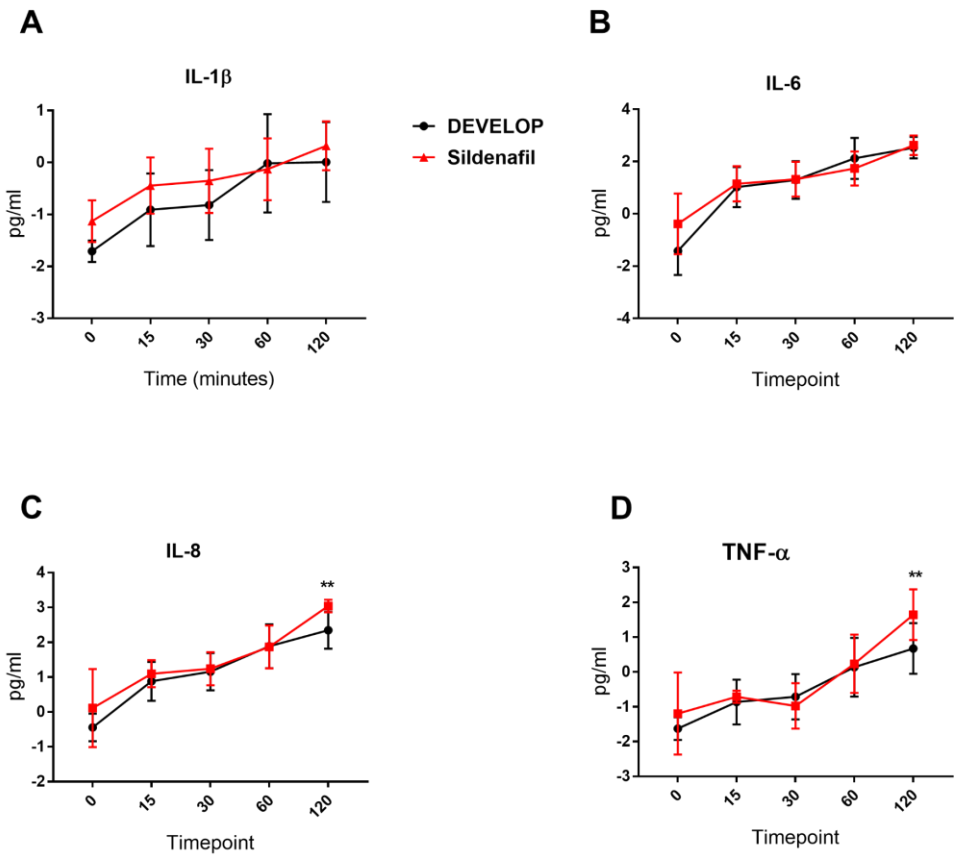

Figure 5.4 Comparison of cytokine concentrations between sildenafil and DEVELOP-UK cohorts.

*Perfusate samples were collected from the circuit prior to commencing EVLP, at 15 minutes and then at 30 minute intervals during perfusion. Perfusate concentrations in pg/ml of IL-1β (graph A), IL-6 (graph B), IL-8 (graph C) and TNF-α (graph D) were detected using a MSD Multi-Array® U-PLEX Human Biomarker pro-inflammatory Panel 1 Kit (K15067L-1) (Meso Scale Diagnostics, LLC, Rockville, MD), according to manufacturer instructions. The DEVELOP-UK historical cohort was used as a comparative group. Data shown as mean +/- SD, analysed by multiple unpaired t-tests, with a post-test correction for multiple comparisons (Bonferroni) **P<0.01. Sildenafil group n=5, DEVELOP group n=46. There were no significant differences seen in IL-1ß and IL-6 concentrations detected in perfusate between groups. In the sildenafil group there were significantly higher levels of IL-8 and TNF-alpha by the end of perfusion.*

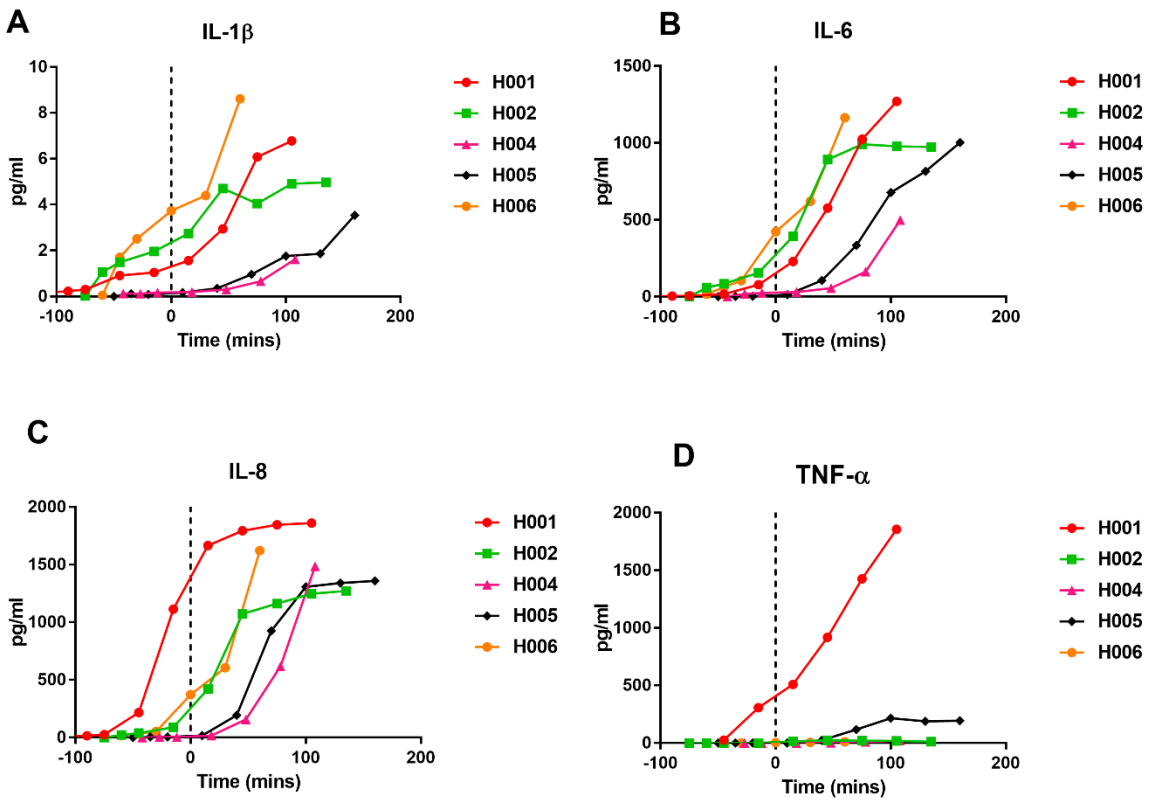

Figure 5.5 Effect of sildenafil on cytokine expression in perfusate.

In all lung pairs shown, the selective PDE-5 inhibitor sildenafil (Revatio; Pfizer, Sandwich, Kent) at a dose of 10 mg was administered as a bolus into the arterial arm of the EVLP circuit after a period of stable reperfusion, 30 minutes of ventilation and when the lungs had reached 37 °C. The black dotted line indicates time 0, when sildenafil was given. Perfusate samples were collected from the circuit prior to commencing EVLP, at 15 minutes and then at 30 minute intervals during perfusion. Perfusate concentrations of IL-1β (graph A), IL-6 (graph B), IL-8 (graph C) and TNF-α (graph D) were detected using a MSD Multi-Array® U-PLEX Human Biomarker pro-inflammatory Panel 1 Kit (K15067L-1) (Meso Scale Diagnostics, LLC, Rockville, MD), according to manufacturer instructions. Data analysed using repeated measures ANOVA, n=5. There is a linear trend for all markers, no significant differences were detected following sildenafil administration.

Effect of sildenafil on endothelial marker expression

When comparing the concentrations of vascular endothelial markers E-selectin, Thrombomodulin, VE-cadherin and von Willebrand Factor in the perfusate of the 5 donor lungs that received sildenafil versus the historical DEVELOP-UK cohort[98] there were significant differences seen. Using multiple t-tests and bonferroni corrections to compare groups at each time point, there were significantly higher levels of Thrombomodulin and VE-cadherin in the DEVELOP-UK cohort at all-time points ($p<0.01$). There was a higher concentration of von Willebrand Factor in the DEVELOP-UK group at 120 minutes, ($p<0.01$). The comparison of the endothelial marker levels between groups can be seen in **Figure 5.6**. The effect of sildenafil on endothelial marker protein levels can be seen in **Figure 5.7.**

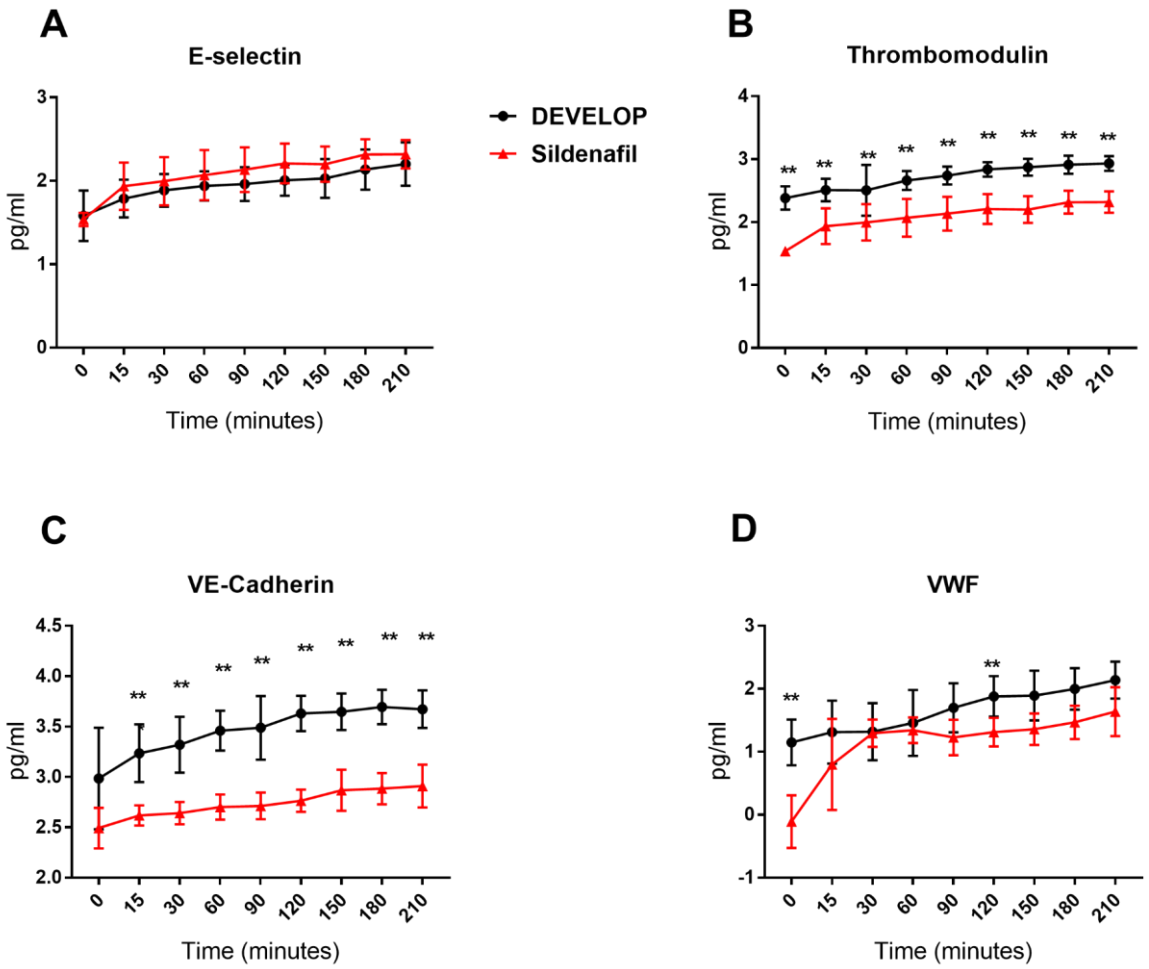

Figure 5.6 Comparison of endothelial marker concentrations between sildenafil and DEVELOP-UK cohorts.

*Perfusate samples were collected from the circuit prior to commencing EVLP, at 15 minutes and then at 30 minute intervals during perfusion. Perfusate concentrations of E-selectin (graph A); Thrombomodulin (graph B); VE-cadherin (graph C) and Von willebrand (VWF) (graph D) were detected using solid phase sandwich ELISAs (R&D Systems, Inc., Minneapolis, MN), according to manufacturer instructions. The DEVELOP-UK historical cohort was used as a comparative group. Data shown as mean +/- SD, analysed by multiple unpaired t-tests, with a post-test correction for multiple comparisons (Bonferroni) **P<0.01. Sildenafil group n=5, DEVELOP group n=46. There were no significant differences seen in E-selectin concentrations detected in perfusate between groups. In the DEVELOP-UK group there were significantly higher levels of THR and VE-cadherin throughout perfusion. VWF was significantly higher in the DEVELOP-UK group at 120 minutes.*

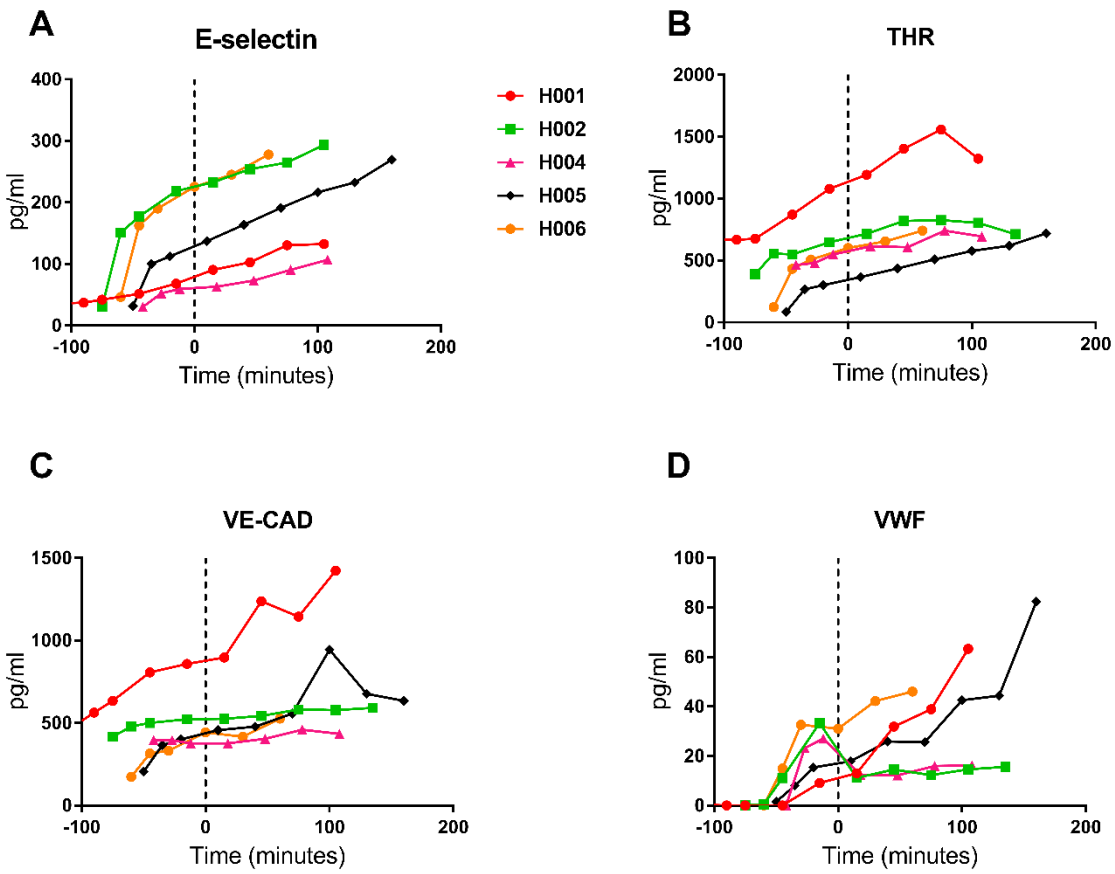

Figure 5.7 Effect of sildenafil on endothelial marker expression

In all lung pairs shown, the selective PDE-5 inhibitor sildenafil (Revatio; Pfizer, Sandwich, Kent) at a dose of 10 mg was administered as a bolus into the arterial arm of the EVLP circuit after a period of stable reperfusion, 30 minutes of ventilation and when the lungs had reached 37oC. The black dotted line indicates time 0, when sildenafil was given. Perfusate samples were collected from the circuit prior to commencing EVLP, at 15 minutes and then at 30 minute intervals during perfusion. Perfusate concentrations of E-selectin (graph A); Thrombomodulin (THR) (graph B); VE-cadherin (graph C) and Von Willebrand (VWF) (graph D) were detected using solid phase sandwich ELISAs (all R&D Systems, Inc., Minneapolis, MN). according to manufacturer instructions. The dotted black line indicates the time at which sildenafil was administered. Data analysed using repeated measures ANOVA, n=5. There is a linear trend for all markers.

Effect of sildenafil on lung histology

The expression of eNOS and neutrophil elastase in immunohistochemistry stained EVLP donor lung tissue was evaluated. Paraffin embedded lung tissue from four of the EVLPs at four time points (pre, 30 minutes after sildenafil, 60 minutes after sildenafil and post) were stained for endothelial nitric oxide synthase (eNOS)and neutrophil elastase (NE). We aimed to investigate if following the administration of sildenafil there was an increase in eNOS and a reduction in NE on parenchymal endothelial cells of lungs having undergone the EVLP assessments. Representative images of low intensity and high intensity eNOS stained pulmonary vascular endothelium are displayed in **Figure 5.8**, with individual scoring in **Figure 5.9.**

Low expression **High expression**

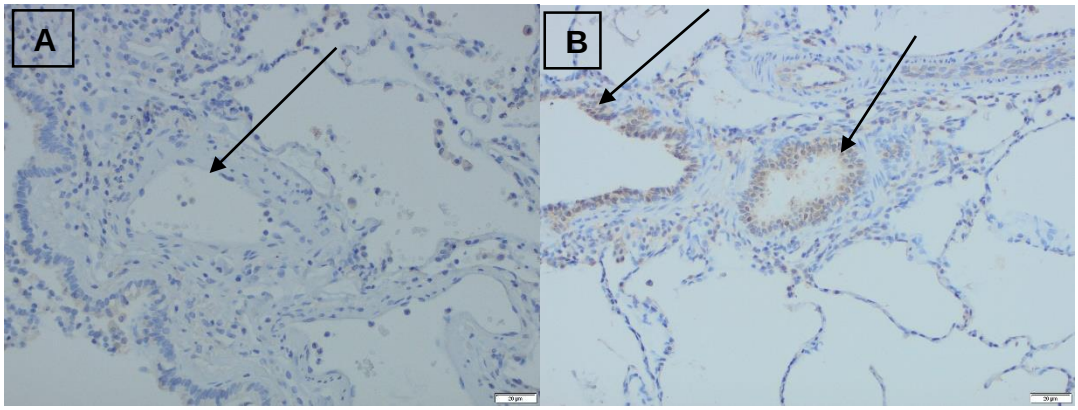

Figure 5.8: eNOS stained lung tissue

Representative examples of digital photos at x20, expressions of eNOS in immunohistochemistry stained EVLP donor lung tissue. Paraffin embedded donor lung sections DAB stained for eNOS expressions. Arrows marking the low intensity stained (A) or high intensity (B) stained pulmonary vascular endothelium.

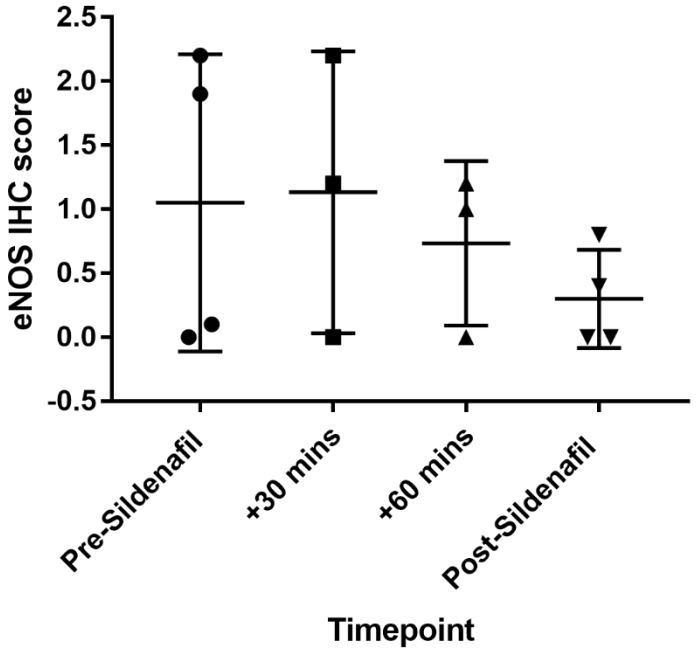

Figure 5.9 Scoring of in eNOS EVLP Tissue

Individual eNOS scores for lung tissue taken before commencing EVLP, 30 minutes after sildenafil was given, 60 minutes after sildenafil was given and after stopping EVLP. Five fields were captured at x20 magnification from each time point section and were independently scored. The vascular endothelial staining was graded using a semi-quantitative scoring system with 4 levels: (0 = no staining; 1 = weak staining, 2 = moderate staining; and 3 = strong staining) Average scores were used and compared for the different time points. There were no significant differences when comparing each time point using one-way ANOVA. The overall trend showed that eNOS staining fell during perfusion with no increase following sildenafil.

There was no significant difference in eNOS expression between the time points. We did not see the expected increase in eNOS following the delivery of sildenafil to the EVLP circuit.

Neutrophil elastase (NE) is a specific stain used in the lung biopsy samples to examine the numbers of neutrophils in the pulmonary vasculature. IHC staining demonstrated that there was a reduction in NE between the pre and post sildenafil biopsies, **Figures 5.10 and 5.11.**

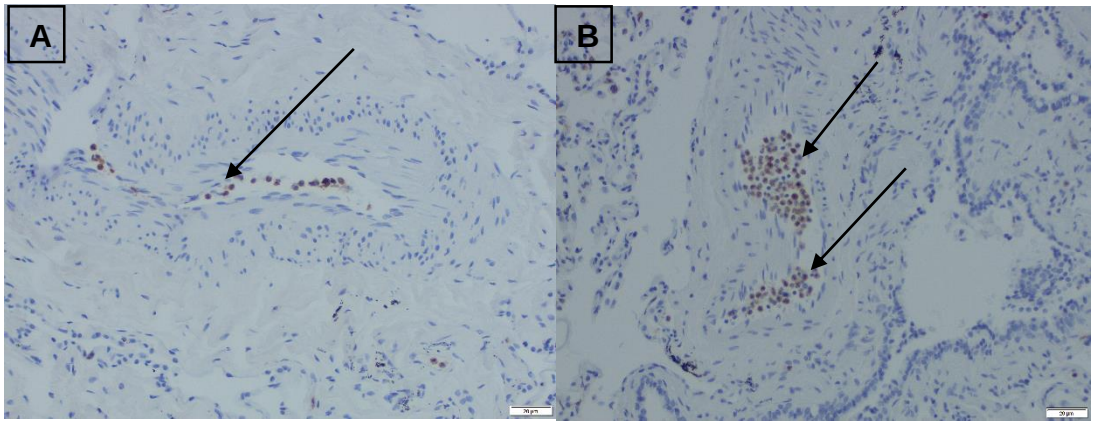

Figure 5.10: Neutrophil elastase stained lung tissue

Representative examples of digital photos at x20, expressions of neutrophil elastase (NE) in immunohistochemistry stained EVLP donor lung tissue. Paraffin embedded donor lung sections DAB stained for NE expression. Arrows marking neutrophil elastase stained cells. A. shows neutrophil elastase cells adhered to the pulmonary vascular endothelium. B. shows neutrophil elastase cells within a pulmonary vessel.

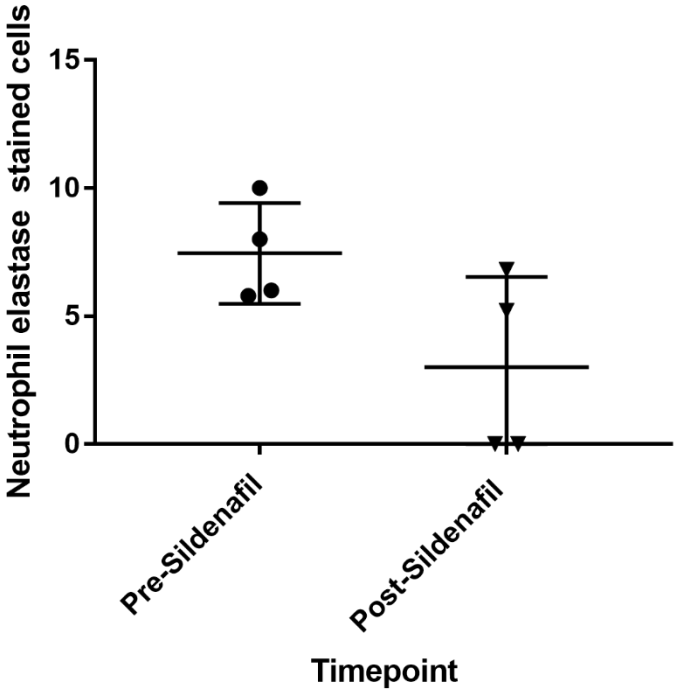

Figure 5.11 Scoring of Neutrophil Elastase in EVLP Tissue

Neutrophil Elastase positive cell count before and after EVLP. The overall trend shows a reduction in the neutrophil elastase stained cell count, however this did not reach statistical significance. Data analysed using Mann-Whitney U test, (p=0.1143).

Discussion

In this study, we examined the effects of administering sildenafil to the vascular compartment of donor lungs during EVLP. We found no measurable differences in lung physiology after sildenafil administration. We did, however, observe significantly lower expression of vascular endothelial activation markers when comparing to a historical control cohort.

The principal mechanism of action of sildenafil involves modulation of NO-activated cGMP by directly inhibiting the PDE-5 enzyme responsible for cGMP breakdown. The resulting increase in cGMP leads to the activation of protein kinase G (PKG), which phosphorylates downstream targets including receptors, kinases, and phosphatases leading to a variety of cellular effects. PKG can, in turn, phosphorylate both enzymes endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), leading to feedback regulation of NO production[203-206]. Increased PKG leads to pulmonary vasodilation and to the opening of potassium channels on the inner mitochondrial membrane which can contribute to the cellular stabilisation. Increases in cGMP and eNOS may also mediate platelet aggregation, neutrophil and leucocyte adhesion[207-209]. Studies have also suggested that delayed protection may result from PDE-5 activation of the Bcl-2 gene, shown to inhibit apoptosis[204].

NO released from endothelial cells maintains vascular homeostatic properties[210] by relaxing vascular smooth muscle[211], inhibiting neutrophil adhesivity[201, 212], platelet aggregation[213, 214]and maintaining endothelial barrier properties[209, 215]. In the lungs, endogenously produced NO stimulates basal cGMP production and results in pulmonary vascular tone.

Considerable interest has therefore been directed towards strategies that supplement the NO/cGMP pathway in an effort to attenuate IRI. The administration of the PDE-5 inhibitor, sildenafil during EVLP in this study did not improve lung physiology or attenuate endothelial marker and cytokine expression.

Our data indicates that although pulmonary vascular resistance was improved following the administration of sildenafil, there were no improvements in physiological parameters such as pulmonary artery pressures, pulmonary flow rates or oxygenation capacity. These results are in contrast to those that have that have reported that endogenous NO levels plummet rapidly after reperfusion[216] and that exogenous NO has been reported to be protective in many models of IRI[217, 218].

NO is responsible for controlling many biologic processes. NO has a key role in regulating blood flow and maintaining vascular tone. NO has a role in several important physiological processes, including vasoregulation and immunomodulation[219]. It is produced by a family of enzymes, i.e., NOSs that catalyses the conversion of L-arginine to L-citrulline with the help of five cofactors. NO then stimulates soluble guanylyl cyclase, which catalyses the formation of cyclic 3′-5′-guanosine monophosphate, which in turn regulates protein phosphorylation, ion channel conductivity, and phosphodiesterase activity. We demonstrated that following the delivery of sildenafil to the EVLP circuit, increased concentrations of nitrite, the breakdown product of NO metabolism were detectable in the perfusate.

Multiple approaches have been utilised to compensate for the fall in endogenous NO during lung transplantation. Strategies have been targeted at the donor and/or to the recipient and have focused on each step of the pathway described previously. Strategies in the literature, have included the administration of the upstream precursor molecule L-arginine[220], methods to increase the downstream effector molecule cyclic 3′-5′-guanosine monophosphate[221, 222], and the administration of exogenous NO. Exogenous NO has been given directly by inhalation (inhaled NO)[218, 223-227] or indirectly by infusion of a NO donor, such as nitroprusside[228], glyceryl trinitrate[229] or nitroglycerin[230-233].

The role of NO as an anti-inflammatory and anti-adhesive modulator in post-ischaemic venules of various organs has been studied. Kurose *et al.* [207]studied leukocyte adherence and emigration as well as albumin extravasation in single post-capillary venules in rat mesentery subjected to 20 minutes of ischaemia followed by 30 minutes of reperfusion. NO donors (sodium nitroprusside, spermine-NO, and SIN1) significantly reduced the IRI-induced leukocyte adherence, emigration and albumin leakage in post-capillary venules. Platelet-leukocyte aggregation and mast cell degranulation were also observed in the post-ischaemic mesentery, and the responses were also attenuated by the NO donors. NO is a homeostatic regulator of leukocyte adhesion in the microcirculation. Inhibition of NO production leads to increased leukocyte rolling and adhesion in various vascular beds and two adhesion molecules, P-selectin and CD11/CD18, have been implicated in these processes.

Other animal studies have demonstrated the effects of inhaled NO on IL-8 release[234]. Gomez *et al.* studied 22 pigs randomly assigned to NO-treated and control groups. In NO-treated pigs, NO at 20 ppm was administered 30 min before

harvest. Pre-treatment with NO at the time of harvesting showed improvement of allograft function in terms of dynamic and static compliance and gas exchange. They also demonstrated that in the inhaled NO group there was a significantly diminished IL-8 release. Our data did not suggest any difference in cytokine expression between our sildenafil group and historical control.

Several clinical studies involving lung transplant recipients have demonstrated the beneficial effects of NO in IRI. Egan *et al.* reported that the administration of the nitric oxide-donor, nitroglycerin to lungs would reduce IRI by activation of cGMP and by reducing capillary leak after reperfusion[235].

Date *et al.* demonstrated that nitric oxide improves oxygenation and decreases pulmonary artery pressure without systemic circulatory effects in patients with severe allograft dysfunction[236].

Moreno *et al.* investigated 32 lung transplant recipients who were randomised to the inhaled NO (iNO) treatment or the control group[237]. Patients in the first group were given 10 ppm of iNO from the beginning of the lung transplant until 48 hours afterwards. The iNO treatment group showed a lower incidence of PGD (29%) in comparison with the control group (40%). Significant differences ($P < 0.05$) were observed in the iNO group, with lower IL-6 levels at 12 hours in blood and BAL. A lower percentage of IL-8 was also detected in the iNO group at 24 hours in BAL and 12 hours in blood and BAL. They concluded that iNO appeared to modulate the inflammatory response by reducing inflammatory cytokine concentrations found immediately after re-implantation, and this reduction was related to a lower incidence of PGD.

Nitric oxide itself has been demonstrated to mitigate IRI in many model systems. However, studies examining the inhalation delivery of nitric oxide in experimental and clinical lung transplantation have shown discrepant results; some show benefits[219] others, however, demonstrate detrimental or neutral effects[238]. NO donors have been shown to be beneficial during the reperfusion period by augmenting the bioavailability of NO after ischaemic injury. They are, however, limited by their rapid release and potentially toxic side effects[239].

Botha *et al.* performed a study of 20 bilateral sequential lung transplant recipients who were randomised to receive either 20-ppm inhaled NO or a standard anaesthetic gas mixture from the onset of ventilation[240]. Bronchoalveolar lavage was

performed immediately prior to implantation and after 30 minutes of reperfusion and analysed for inflammatory cytokine levels and free radical surrogates. Primary graft dysfunction (PGD) scoring was performed prospectively for 72 hours' post-transplant. The study could not demonstrate a significant effect of inhaled NO during the first 30 minutes of reperfusion in the prevention of neutrophil injury and primary graft dysfunction after lung transplantation.

Ardehali and colleagues have shown that the application of inhaled NO to 28 consecutive recipients after lung transplantation did not prevent the occurrence of primary graft failure[241].

In a prospective, randomised controlled study involving 30 double lung transplant recipients, Perrin *et al.* illustrated that prophylactic use of iNO failed to decrease pulmonary oedema formation and resolution after pulmonary reperfusion[242].

The presence of NO and simultaneous generation of superoxide during reperfusion may lead to the formation of peroxynitrite ($ONOO^-$), a powerful pro-oxidant that may enhance graft injury[243-246]. $ONOO^-$ catalyses membrane peroxidation and reacts to form toxic nitrosylating species. Studies suggest that NO can be either protective or toxic to lung grafts depending on dose, timing, duration of NO administration, the source of NO and the local re-dox environment and indicate that there is a narrow therapeutic window for NO in lung transplant IRI[247].

Eppinger *et al.* demonstrated in a rat lung model that the injury during the first 4 hours of reperfusion is bimodal in pattern, with significant injury peaks occurring at 30 minutes and 4 hours, with partial recovery after the 30-minute time point suggesting that the two peaks of injury are separate. This group went on to show that the delivery of inhaled NO at the start of reperfusion markedly worsened injury at 30 minutes and that the increase in injury was due to an interaction with endogenously produced superoxide. Delaying the delivery of NO for 10 minutes, after the early burst of superoxide production had passed, eliminated the increase in injury. This group showed that at 4 hours of reperfusion the beneficial effects of inhaled NO could be seen with a reduction in lung vascular permeability. They found that the neutrophil accumulation in lungs at 4hrs reperfusion was less in the NO treated group than controls. NO is known to decrease the adhesive interactions between neutrophils and endothelial cells via their expression of the adhesion molecule CD11/CD18[201].

Our study identified significant differences in the expression of endothelial markers shed into perfusate between our sildenafil group and the historical control group. Higher levels of thrombomodulin, VE-cadherin and von Willebrand factor were detected in the control group. This postulates whether sildenafil has a protective effect on the vascular permeability.

The pulmonary endothelium is formed from a monolayer of mesenchymal-derived squamous endothelial cells[192] connected by junctional proteins, forming a semi-permeable barrier between the circulation and pulmonary interstitium. It prevents vascular leak by forming a physical barrier. When the integrity of this barrier is compromised, there is increased permeability and fluid shift out of the intravascular space. Junctional proteins are vital in maintaining the integrity of the endothelial barrier. Adherens junctions are the most populous junctional proteins in the endothelium and are predominantly vascular endothelial cadherin (VE-cadherin) based. VE-cadherin down-regulation and shedding is induced by thrombin presence[248] and oxidative stress[249] and is associated with pulmonary oedema formation and respiratory distress[250]. VE-cadherin loss has previously been demonstrated in areas where endothelial integrity has been comprised after cold ischaemia[251].

von Willebrand factor (vWF), a pro-thrombotic, is found in weibel-palade bodies in endothelial cells[252]. Activated pulmonary endothelial cells release vWF by two independent mechanisms calcium dependent in the presence of thrombin, or cAMP dependent in the presence of epinephrine or adenosine[253, 254]. Calcium-dependent vWF release is activated in the presence of thrombin and results in GTPase Rho-associated microtubule dissolution and subsequent redistribution of actin and myosin into dense stress fibres. This causes endothelial cell retraction and reduced barrier integrity[255]. This process is rapid and can occur within 20 minutes of exposure to thrombin[253]. Once released, vWF attaches to platelets via platelet glycoprotein 1b, initiating platelet plug formation[252]. vWF increases leukocyte extravasation independently of leukocyte adhesion and rolling. This suggests vWF has a direct role in increasing endothelial permeability[252, 256], though this has not been demonstrated on pulmonary endothelium.

Evidence on the clinical relevance of vWF release in pulmonary oedema is conflicting. Increased serum levels of vWF have been associated with acute lung injury and pulmonary oedema[257]. vWF serum levels are a poor prognostic indicator

for survival in acute lung injury[258]. Conversely, other work found no significant association between serum vWF, e-selectin or thrombomodulin and protein accumulation with the lung (a surrogate for pulmonary endothelial permeability)[259]. In lung transplantation, serum vWF in recipients was not associated with PGD[260].

As described above, thrombin is an oedematogenic agent. When thrombin is co-activated by thrombomodulin and endothelial protein kinase C (PKC) receptor from the surface of endothelial cells, there is activation and release of PKC, which cleaves activated clotting factors V and VIII[261].

We did not see any significant difference in the staining of eNOS when comparing lung tissue at different time points during EVLP, in relation to when sildenafil was given. It was expected that eNOS would increase following the drug delivery. We did not take a sample immediately before delivery, and the pre biopsy was taken prior to EVLP commencing and therefore may only reflect the effect of perfusion itself. In future studies, it would be helpful to take a biopsy prior to commencing EVLP and immediately before drug delivery.

It may be that the biopsy taken 30 minutes after drug delivery was too late to see an effect on eNOS and that an earlier 5-minute biopsy would be helpful. In future studies, it would be helpful to measure iNOS in relation to EVLP and the effect of Sildenafil.

Naka *et al.* used a rat lung transplantation model showing that inhaled NO treatment during reperfusion improved function in only 4 of 12 grafts whereas addition of a cGMP analogue to the storage solution was uniformly beneficial[262]. They demonstrated that the addition of a membrane permeable cGMP analogue to the preservation solution resulted in the stabilsation of pulmonary haemodynamics after transplantation and attenuated graft neutrophil infiltration. The authors concluded that the former treatment was more beneficial because it bypassed the reactive oxygen species step. Injury in rat lungs undergoing 90 minutes of warm *in situ* ischemia was significantly worsened when inhaled NO was administered from the onset of reperfusion[263]. This effect was reversed by addition of superoxide dismutase or by delaying inhaled NO therapy for 10 minutes. The authors hypothesised that the interaction of NO with the burst of superoxide generated at reperfusion leads to the production of toxic peroxynitrite and hydroxyl radicals and may be responsible for these observations.

It is therefore believed that distal stimulation of the NO/cGMP pathway at the point of cGMP might confer the beneficial vascular effects of NO while avoiding its potential toxicities. Inhaled NO facilitates ventilation-perfusion matching as it is inhaled. By definition, vasodilation induced by inhaled NO occurs in areas that are being ventilated. In contrast, the distal simulation of the NO pathway with soluble mediators is independent of ventilation.

The effects of stimulating the NO/ cGMP pathway at the point of cGMP have been investigated. Pinksy *et al.* showed that NO availability is sharply diminished after preservation and reperfusion of the lungs, resulting in a fall in tissue cGMP levels[221]. They investigated whether replenishing this intracellular second messenger would enhance graft vascular function. Using a rat lung transplant model with the addition of 8-Br-cGMP to the preservation solution used during reperfusion. Supplemental 8-Br-cGMP increased PA flow, increased P02 and decreased PVR. They concluded that augmenting the NO pathway at the level of cGMP provides a useful pharmacological approach to normalising vascular function in the early stages following pulmonary ischaemia.

In addition to enhancing levels of cGMP that result in vasodilation and enhanced tissue oxygenation, cGMP is also known to facilitate other cellular processes through the activation of PKG. This is thought to promote protein phosphorylation, cell growth, and survival giving protection against cellular injury[206, 246]. Cyclic GMP is also known to inhibit platelet activation, to have anti-apoptotic properties, and to reduce production of reactive oxygen species[264].

Sandera *et al.* found that substitution of the NO pathway by administration of 8-Br-cGMP, a membrane permeable analogue of cGMP, the second messenger of NO at the time of reperfusion improves post-transplant lung allograft function[222].

In recent years, there has been considerable interest in studying the effect of sildenafil and other PDE-5 inhibitors in protection against IRI[265, 266].

Das *et al.* demonstrated activation of protein kinase C could be one of the intracellular signal transduction pathways controlling sildenafil dependent cardiac protection in the rabbit heart[267]. A direct action of sildenafil on mitochondrial K-ATP channels has also been suggested to mediate the sildenafil-induced protection against ischemic injury.

Lui *et al.* studied the protective effect of the sildenafil on lung IRI[268]. They studied 30 rats which were randomly divided into 3 groups of 10: a sham-operated group A, a lung ischemia-reperfusion injury group B, and a sildenafil preconditioned group C. Compared to group B, the lung wet/dry ratio, malondialdehyde content, myeloperoxidase and nitric oxide synthase activity in group C were significantly lower, while arterial PO2 and cGMP content in group C were significantly higher. They concluded that preconditioning with sildenafil prevented rat lung IRI and improved pulmonary function.

Korom *et al.* demonstrated that restoring intracellular cGMP and inducing NO-synthesis attenuates ischemia-associated early pulmonary allograft dysfunction[269]. They treated donor pigs with intravenous sildenafil injected directly into the PA before experimental lung transplantation. Pigs that received sildenafil had improved short-term survival. This group concluded that by administering sildenafil during lung perfusion, storage and implantation, ischaemic tolerance may be extended and early graft function improved.

Weiss and colleagues demonstrated that pre-treatment with the long-acting PDE-5 inhibitor, tadalafil, could significantly reduce IRI in a rabbit lung transplantation model. This effect was attributed to the increasing tissue content of cGMP and the decreasing production of ROS[10].

Kiss *et al*. reported that in a rat pulmonary hypertension model, sildenafil suppressed multiple cytokines involved in neutrophil and mononuclear cells recruitment including cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-2a/b, tissue inhibitor of metalloproteinase (TIMP)-1, interleukin (IL)-1a, lipopolysaccharide induced CXC chemokine (LIX), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1a, and MIP-3[200]. They suggest a beneficial effect of sildenafil on inflammatory mechanisms that substantially contribute to its protective effects.

Shih *et al.* demonstrated that pre-treatment with sildenafil reduced TNF-α and IL-6 levels of lung tissue, concluding that sildenafil preconditioning improves lung inflammation in early reperfusion injury in a rat model[270].

The effect of sildenafil on attenuating ischaemia-reperfusion injury has also been demonstrated in other solid organ transplant models. Savvanis *et al.* evaluated the effect of sildenafil in a liver IRI rat model and tested the hypothesis that sildenafil

exerts a protective effect on the liver[271]. Serum markers of hepatocellular injury were significantly lower in the sildenafil group, which also exhibited lower severity of histopathological lesions, decreased scores of necrosis, attenuation of morphological liver injury, and anti apoptotic activity, as compared to the control group. The control group showed significantly higher leukocyte-endothelial interaction as evidenced by MPO activity and higher expression of ICAM-1, as compared to the sildenafil group.

Lledo-Garcia *et al*. studied the protective effects of sildenafil on kidney grafts auto transplanted after 45 minutes of warm ischaemia by vascular clamping, nephrectomy and 60 minutes of isolated hypothermic pump perfusion. They found significantly higher renal vascular flow rates and lower renal vascular resistance in the sildenafil group.

The use of sildenafil as a preconditioning agent predominates the evidence in treating ischaemic injury. There is evidence that sildenafil is capable of inducing a preconditioning-like effect in IRI of various tissues such as the heart, lung, kidney, and brain. Botha *et al.* investigated the use of sildenafil-mediated cardio protection in a rat model of heterotopic cardiac transplantation[272]. They concluded that sildenafil pre-treatment augments myocardial functional recovery after an ischemic time relevant to clinical cardiac transplantation. They linked this with protein kinase C activation/translocation and inhibited by 5-hydroxydecanoate.

Du Toit *et al.* reported that the pre administration of sildenafil increased NO levels by the stimulation and opening of mitochondrial K+ATP channels[273, 274].

Kukreja and colleagues[204] reported a myocardial protective effect of sildenafil preconditioning. The mechanisms included the opening of the mitochondrial adenosine triphosphate-sensitive potassium channel, reducing the intercellular calcium overload, and improving endothelial function.

Hillinger *et al.* demonstrated that 8-Br-cGMP (a membrane-permeable analogue of cGMP) when used as an additive to the flush solution, improves post-transplant lung oedema, lipid peroxidation, and neutrophil migration to the allograft[275]. Sildenafil as a potent vasodilator may improve the distribution of hypothermic flush solution.

Pizanis *et al.* investigated the effect of adding sildenafil to the procurement procedure of porcine lungs[276]. They studied 3 groups which allowed for different application procedures to be investigated: a control group, a group flushed with Low Potassium

Dextran (LPD) with 0.15 mg/kg sildenafil added and a third group which were preconditioned with an intravenous bolus of 0.15 mg/kg of sildenafil into the pulmonary artery 20 minutes prior to the standard control LDP flush. The group which was preconditioned with the sildenafil IV bolus demonstrated lower PAP and PAP, superior oxygenation after 6 hours of reperfusion.

This study has several limitations. The ex vivo reperfusion model allows us to study the effect of ischaemia reperfusion injury in the isolated lung pair without an immunologic host response. One potential drawback of EVLP, is that we are unable to study long-term effects. As we reperfused for less than 4 hours, the effects we have observed with PDE-5 inhibition may be short-lived, and the effects of this therapy in the longer term are unknown.

A high dose of sildenafil was chosen for maximum effect and was administered at the onset of reperfusion. Adding therapies to the perfusate solution allows a high concentration of a drug to be administered without any adverse effects. We based our dosage regime on human dosing in the context of pulmonary hypertension and literature available, however we do not know whether a more optimal dose could have been used, whether an infusion strategy would have been better than a bolus dose, the timing of administration could have been improved and whether a bolus dose should have been repeated.

It must be noted that the EVLP protocol used in this study differs from the protocols used in DEVELOP-UK, the historical cohort. It is not known what impact the differences between protocols, most importantly 40% versus 100% cardiac output, open versus closed left atrium and acellular versus cellular, had on the data interpretation. One may argue that the acellular study used in our study was somewhat less physiological than a cellular perfusate, although literature to support this is lacking.

It is essential to recognise the length and variation in ischaemic times in our study cohort. This was a “real-time” research study which was subject to the logistical time constraints. In the first three EVLPs, the lungs had ischaemic times greater than twenty hours, and this will undoubtedly have an effect on lung function and performance. Although human donor lungs being made available for research are a scare and precious resource, moving forward into future studies, we would consider a twelve-hour ischaemic time to be an upper limit.

Animal models predominate the literature when investigating the role of sildenafil in attenuating IRI and our study aimed to provide a novel platform, in the context of human adult donor lungs. Animal models have certain limitations relating to species differences in susceptibility to ischemia-reperfusion injury, inflammatory responses, and gene regulation. Our study aimed to demonstrate the effects of sildenafil on human donor lungs in the context of EVLP.

In conclusion, IRI remains a substantial cause of morbidity and mortality after lung transplantation. EVLP provides a platform for investigating the effect of novel therapeutics to protect and potentially recondition donor lungs. In this study, we have examined the effect of sildenafil, a PDE-5 inhibitor on pulmonary performance during EVLP and downstream immunological effects. We did not observe any improvements in lung physiology following sildenafil however in this group there were lower concentrations of endothelial markers in perfusate which could indicate a protective effect. Overall, our group concludes that more in-depth investigation should be conducted to explore the exact molecular and genetic mechanisms of the effects of sildenafil in human donor lungs on EVLP, as well as the feasibility and safety of its clinical application.

CHAPTER 6

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Discussion

Chapter 6: Discussion

The research contained within this thesis has been conducted as part of the NIHR BTRU-ODT collaboration, a unique strategic partnership between Newcastle and Cambridge Universities and NHS-BT. Since my project's infancy, being part of such a structured framework has helped guide and steer project aims and objectives. From the outset of the project, there were three clearly defined overarching aims: to develop EVLP as a technique for the assessment of lungs turned down for transplantation; to identify potential biomarkers that will help us to understand the characteristics of human donor lungs and predict the success of EVLP and clinical outcomes and thirdly to use EVLP as a therapeutic platform to treat donor lungs.

Starting my project at Newcastle University in October 2016 marked an important time in the timeline of EVLP research in the UK. The results of DEVELOP-UK had been recently published and involved all five UK NHS adult lung transplant centres. DEVELOP-UK aimed to compare survival during the first 12 months following lung transplantation between EVLP and standard groups. At the time of starting my project, DEVELOP had demonstrated that EVLP could be used to utilise donor lungs unsuitable for standard transplantation with a conversion rate to transplantation of 18/53 (34%). The main trial outcomes can be summarised below:

- The Kaplan-Meier estimate of survival at 12 months was 0.67 [95% confidence interval (CI) 0.40 to 0.83] for the EVLP arm and 0.80 (95% CI 0.74 to 0.85) for the standard arm.
- Patients in the EVLP arm were seen to require a longer period of ventilation and intensive therapy unit (ITU) length of stay, than patients in the standard arm, but the duration of overall hospital stay was similar in both groups.
- These differences are most likely explained by the higher rate of early grade 3 primary graft dysfunction (PGD) in the EVLP arm. Rates of PGD did not differ between groups after 72 hours.
- The requirement for extracorporeal membrane oxygenation (ECMO) support was considerably higher in the EVLP arm (7/18, 38.8%) than in the standard arm (6/184, 3.2%).
- DEVELOP-UK reported on the cost of EVLP transplants as approximately £35,000 higher than the cost of standard transplants.

DEVELOP-UK was a colossal body of work that was an achievement of the UK lung transplant community. A number of questions arose from its results, and several of these remain unanswered. DEVELOP-UK created a vast number of clinical samples which had been collected, processed and stored under the same protocol. This has created a unique biobank of EVLP specimens which hold the answers to many research questions. My project has benefitted from access to those samples, a unique resource.

I have also benefitted from the local expertise, knowledge and existing equipment that followed the trial. DEVELOP-UK did highlight the substantial costs associated with EVLP, due to the expense of the equipment and consumables. One of the major priorities for my project was to develop a research EVLP model that had been scaled down on cost.

My research project aimed to utilise the knowledge, experience and momentum that came from DEVELOP-UK whilst answering new research questions. To carry out this project, I used two main work streams. The first was the retrospective processing and analysis of DEVELOP-UK samples within my first year. This gave me an introduction to the protocols used in the trial and gave me my first experience of laboratory techniques such as ELISA. This retrospective work proved essential, given the delays that were encountered in finalising approvals for the BTRU lung project.

Following final approval and training in porcine EVLP, we were able to accept human donor lungs which had been turned down for transplantation into the project. This prospective work has allowed for the study focussed on sildenafil as a novel therapeutic in EVLP. It has also generated a large bank of perfusate, BAL and lung tissue samples which are made available to other researchers via the Newcastle Institute of Transplantation Tissue Biobank.

Over the course of this work, a robust, reproducible and cost-effective research EVLP model has been developed. A porcine model was initially developed to provide education and hands-on training in dissection, suturing and circuit set-up. The porcine model also allowed for optimisation of assessment and sample collection protocols. Following the porcine work, we were able to accept human donor lungs which were deemed unsuitable for standard transplantation into our study.

If EVLP is to realise its potential as an innovative platform for the assessment and treatment of donor lungs prior to transplantation, it is essential that the cellular and

molecular events that occur during EVLP are fully understood. Immune profiling in lung transplantation has previously relied on downstream protein production and cellular analysis. If the purpose of the biomarker discovery is to detect the onset of ischaemic injury before an uncontrollable immune activation takes place, then as a method of immune profiling, RNA-Seq transcriptomic analysis illuminates not only individual genes, but entire pathways and upstream regulators. RNA sequencing can provide a fuller picture of the underlying pathology and allow for identification of potential upstream targets to prevent injury before the onset of significant inflammation. Several investigators have shown that brain death and ischaemia-reperfusion injury have a significant impact on complex gene expression changes [37, 136, 137].

In Chapter 3, we demonstrated that the process of EVLP itself induces a significant effect on the lung tissue transcriptome. This is novel information not previously discussed in the literature. Lung transcriptomics, as a method of assessing donor lungs that have undergone EVLP, has before now, not been described in the literature. Kang *et al.* used global gene expression profiling to identify differences between DBD and DCD donor lungs before transplantation and also analysed changes in the gene signature in lung tissue before and after transplantation[139]. Kang *et al.* reported that hundreds of genes and over 30 gene sets were significantly altered during transplantation. In our study, EVLP was associated with the significant upregulation of 297 and downregulation of 86 specific genes. Our study demonstrates that RNA sequencing can be used in the EVLP setting and adds to the existing knowledge of how the donor lung gene signature is altered during ex-vivo perfusion.

We found that the gene pathway that was most significantly upregulated was TNFα Signalling via NFκB and that those donor lungs which performed poorly and went onto fail EVLP assessments had higher upregulation of IFN-γ and TNF-NFκB than lungs that passed. This supports the previous literature published by the Newcastle team, Andreasson *et al.* that perfusate IL-1β levels were strongly associated with early graft performance and could in the DEVELOP-UK multicentre study with high sensitivity and specificity predict both EVLP assessment failure and early post-transplant mortality. Our study showed that inflammasome activation genes are enriched in pre-perfusion biopsies in “fail” lungs shows that in lungs that went on to fail, there was clearly upregulation of the IL-1β genetic signal prior to commencing perfusion.

Following this unbiased approach of identifying genes that were only upregulated in fail lungs with poor outcome, we found that there were 5 genes significantly upregulated in only fail lungs and that these genes corresponded to pro-inflammatory cytokines MIG and MIP-3β; the acute phase proteins PTX3, α_1AGp and HSP-70. We then investigated the protein expression in perfusate for these specific pro-inflammatory and tissue injury proteins of interest. We then compared the levels of cytokine expression between the clinical outcome and protocol groups to assess if we could determine any clinical correlations.

Surprisingly, although there was a trend throughout all five protein markers of higher protein expression in the fail lung group compared with the pass lung group, no time-points reached statistical significance. We had expected to see significant differences in the protein levels of interest between clinical outcome groups as had been observed with IL-1β and TNF-α[83].

We demonstrated that there were higher levels of MIG and MIP-3β concentrations in the perfusates of lungs that underwent the *Hybrid* compared to the *Lund* EVLP protocol. Following DEVELOP-UK, questions had been answered about the impact of changing protocols during the trial. Our work shows that there are increased levels of these pro-inflammatory cytokines during *Hybrid* EVLP where a 40% flow rate, open left atrium and acellular protocol was used. Further work is required to identify reasons for this, to establish if the higher levels of inflammation seen, are due to the open left atrium or the acellular perfusate. No previous research has focussed on a head to head comparison of protocols in human EVLP, and given the significant investments from the Steen and Toronto groups in their EVLP machines and corresponding protocols, it is unlikely this will ever take place. Linacre *et al.* investigated whether maintaining a physiologic positive LA pressure during EVLP is protective to the porcine lung[87]. They reported that using a closed left atrium, creates a controlled positive LA during EVLP and leads to significantly less oedema and improved lung physiology compared to the open technique. It is likely that our findings will influence the protocols used for future EVLP studies. It is well documented in the literature that cardiopulmonary bypass and associated equipment, namely a pump, oxygenator and artificial plastic circuit can induce a systemic inflammatory response (SIRS) by causing cellular activation[277]. One may have thought that by having an acellular perfusate as in the *Hybrid* protocol that there would be less of this SIRS response than with a cellular perfusate.

We have demonstrated that important information about the donor lung gene signature can be deduced from lung tissue prior to commencing EVLP. We showed that the RNA counts in tissue taken before EVLP correlated with the concentrations of culprit pro-inflammatory cytokines measured in perfusate throughout the perfusion. Mapping out the genetic pathways that are upregulated during perfusion adds a new dimension to the way in which we could approach biomarker discovery and therapeutic targeting. It not only confirms what we may already know from BAL and perfusate samples but could also add new information about the molecular events occurring in the donor organ.

Several studies have investigated the impact of donor cytokine levels on recipient outcome and demonstrated that there is a direct correlation between increased cytokine levels in BAL and perfusate with worsening lung function post transplantation[43, 157]. In our study, we demonstrated significantly higher levels of MIG and MIP-3β in donor lungs that had undergone EVLP with a Hybrid protocol. Both MIG and MIP-3β are chemokines with MIG attracting dendritic and antigen-B cells where MIP-3β is a known T-cell chemoattractant. Increased levels of these chemokines in donor lungs could result in a heightened recipient T-cell response, accumulation post-transplantation and potentially an increased risk of acute cellular rejection. It has been shown that depletion of donor immune cells from an organ prior to transplantation prevents rejection[278].

EVLP offers a unique platform to study the effects of depleting donor immune cells and leukocytes. Stone *et al*. demonstrated that the early interactions between donor and recipient leukocytes are thought to play a critical role in long-term outcome following transplantation. They reported that EVLP-mediated donor leukocyte depletion impairs early recipient T cell priming without immunosuppression and represents a novel and exciting approach to tackling acute rejection[279].

My work on transcriptomics during EVLP has introduced the EVLP community to this method of assessing donor lungs and correlating the gene signature to clinical outcome. This assessment technique has provided new information about the genetic pathways that are induced during perfusion and has the potential to identify new therapeutic targets. Transcriptomic studies could be applied to any organ, and perfusion platform and comparisons could then be made in future. It would be interesting to compare the genetic pathways that are upregulated during lung perfusion versus perfusion of the kidneys and liver. It may be, that due to the lungs

dual circulation and the ability for alveoli to maintain a degree of oxygenation during the period of cold storage that there is an element of protection against the effects of hypoxia. It may be that with transcriptomic studies these differences could be recognised at a genetic level.

In Chapter 4, we demonstrated that in extended criteria donor lungs which have been turned down for immediate transplantation, there are differential inflammatory and tissue injury marker profiles between DBD and DCD lungs. We found that IL-4, IL-6 and IL-12 were significantly higher in tissue samples from DBD lungs but this was not reflected in the perfusate concentrations. We also found that IL-α and IL-1β were detected in significantly higher levels in the perfusate of DCD lungs at as early as 15 minutes and throughout reperfusion. These observations demonstrate differences in the cytokine expression between DBD and DCD lungs and also in the compartment from which they are released.

Literature comparing the differences between inflammation in DBD and DCD donor lungs at a cellular and molecular level is limited. Kang *et al*.[139] studied the differential gene expression profiles in lungs of DCD and DBD patients and interpreted the differences using functional pathway analysis. Analysis of gene expression profiles showed distinct activation of gene pathways between pre-transplant DBD and DCD human lungs. They found that there were 12 gene sets highly enriched in DBD but not DCD lungs.

Our study generates important discussion about the impact of the type of donor on donor lung inflammatory burden. We postulate that in the DBD lungs, we missed an early peak of production of IL-1α and IL-1β and by the point of organ retrieval this had diminished. Instead, we have seen the rise in mediators, IL-4, IL-6 and IL-12 following the stimulation of the “early response cytokines” IL-1 and TNF-α. Whereas due to the lung insults such as warm ischaemia occurring much closer to the time of retrieval in DCD lungs higher levels of the early response cytokines were elevated. We suggest that events surrounding death upregulate the early response inflammation in DCD donors.

Our work confirms what has been published by other groups with regards to significantly higher levels of IL-6 ($p=0.001$) being measured in pre-transplant samples in DBD compared with DCD lungs[139]. The results from this study are congruent with

the theory that DBD lungs carry a higher inflammatory burden due to the insult of brain stem death[19].

This study provides insight into the potential molecular events in the period prior to retrieval and differences between the two donor groups. Improved knowledge on when and how fast brain death related processes occur and the sequence in which these events take place, will help us to understand the primary triggers of DBD-associated organ injury. A greater understanding of pre-donation events may inform the creation of donor management protocols tailored specifically to the DCD and DBD lung and evaluation strategies that identify organs suitable for transplantation. Further knowledge of the pathophysiological processes occurring in these groups may identify ways to improve donor management and possible therapeutic targets for improving outcomes in lung transplantation.

It is important to highlight that our study did not investigate clinical outcomes in relation to the levels of inflammatory and tissue injury profiles. We can therefore not make any judgments about whether having higher levels of inflammation at the onset of EVLP led to any differences in outcome. Several studies have shown that DBD and DCD lung transplantation outcomes do not differ and emphasise that the DCD donor pool is an underutilised resource which if used can considerably increase the number of donor lungs available for transplantation[16, 145].

Future studies into the differences between these two donor types are needed. It would be interesting to investigate if there are differences between the inflammatory profiles in the recipients of DBD versus DCD donor lungs, tested in serum and BAL.

In theme 1 of the NIHR BTRU-ODT, led by Professors Chris Watson and John Dark, PhD student Poppy Aldam is carrying out important research focussing on improving the understanding of the physiology of donation after circulatory death (DCD). This research will report the physiological changes that occur during the dying process for patients having life support treatment withdrawn. This study will also investigate the inflammatory profiles measured in the DCD donor during the period leading to the withdrawal of treatment and retrieval. It will be important to compare that data with that already found in the EVLP setting to make some valuable conclusions about how timing impacts inflammatory burden. This period has not been subject to intense study and has the potential to have a significant impact on the number and quality of DCD organs donated. Research in the DCD population is limited by the sensitive nature of

the setting at which samples are required to be taken. It is vital therefore to gain as much understanding of pathological processed during DCD donation through means available, and EVLP offers a unique opportunity to study this cohort of donors.

This thesis posed the question of whether there are differences in the inflammatory profiles of DBD and DCD lungs. It is only through improving our understanding of what is happening prior to donation at a cellular and molecular level can we then focus on ways to improve donor management. The lung transplant community is still learning about the DCD donor, and in many countries, there is still apprehension and reservation about utilising DCD donors. My research aims to generate discussion about DCD donation and demonstrating that through the use of EVLP there is much to be learnt about the biology of these two donor types. My work highlighted that the timing prior to the retrieval of organs might be relevant to the inflammatory insult within the organ.

In Chapter 5 we demonstrated the use of EVLP as a platform for novel therapeutics in donor lungs. This is an ever growing area of interest and certainly using EVLP for drug therapies in donor lungs is not a new concept. We reported on the first study of sildenafil in the context of human EVLP. Several studies have suggested an important role for NO signalling pathways mediated by secondary messengers such as cGMP in the maintenance of vascular homeostasis and have been implicated in IRI. cGMP is an important signalling molecule that stimulates smooth muscle relaxation. Models of lung IRI have demonstrated that reduced levels of cGMP[193] after lung reperfusion have been associated with the development of pulmonary hypertension[194],reduced oxygenation,[195]and increased microvascular permeability[189]. PDE-5 inhibitors such as sildenafil prevent the breakdown of NO driven cGMP[196]. cGMP has regulatory roles that stimulate neutrophil degranulation, inhibit platelet aggregation, regulate intracellular calcium levels, and open the mitochondrial K+ATP channels[198, 199]. These mechanisms have a beneficial role in reducing the severity of IRI. Previous studies have reported that sildenafil can suppress multiple pro-inflammatory cytokines which play an important role in neutrophil and monocyte recruitment[200]. It has also been reported that through modulation of the NO/cGMP pathway, the interaction between neutrophils and endothelial cells via adhesion molecules can be attenuated[201].

It was surprising that in our study, we did not see the expected changes in lung physiology, following the administration of sildenafil to the EVLP circuit. One must

consider the relatively normal pulmonary artery pressures of less than 20 mmHg that were recorded in our study lungs. It may be that sildenafil requires a certain threshold of pulmonary vasoconstriction and therefore pulmonary artery hypertension in order to exert its effect. Further studies administrating sildenafil at increasing pulmonary artery pressures may help to establish if this threshold exists. Another concern from our study is that there may be varying degrees of vasoconstriction and vasodilatation at different anatomical sites during EVLP depending on the amount of NO production within the vascular system. This could lead to an area of V/Q mismatch whereby metabolically active lung tissue which is being under-perfused and over-perfusion of metabolically inactive tissue with subsequent shunting, similar to what is seen in septic shock[280]. Pulmonary gas exchange is normally preserved and optimised by a physiological phenomenon called hypoxic pulmonary vasoconstriction which promotes perfusion to areas of ventilated lung instead of under-ventilated areas which are considered “physiological dead space”. One might postulate that interruption with the NO pathway could lead to a disturbance in this pulmonary regulatory mechanism.

When considering possible explanations for the lack of hypothesised effects we need to consider that the efficacy of sildenafil is dependent on the presence of sufficient amounts of endogenous NO to activate soluble guanylyl cyclase (sGC) and generate cGMP. Low endogenous NO/cGMP production significantly limits or impairs the effects of PDE-5 inhibitors. It could be postulated that endogenous NO levels were too low in the donor lung EVLP system for sildenafil to have its desired effects of reducing pulmonary artery pressure and pulmonary vascular resistance. Stimulators of sGC, such as riociguat could be an alternative therapy as this could increase cGMP, independently of NO. Chammaro *et al.* have reported that riociguat was more effective as a vasodilator in isolated rat and human PA than sildenafil[281]. Future studies could test the effect of riociguat in the EVLP model, and this could be tested next to a control and sildenafil group to compare the effects. Sildenafil has been investigated in other clinical areas such as the treatment of pre-eclampsia. Studies in this field provide evidence that the effects of sildenafil are independent of NO whereby the maternal and feto-placental protective effects with sildenafil treatment were not dependent on circulating NO and cGMP levels[282].

In future studies, it would be useful to investigate the effect of sildenafil in human lungs using a paired experimental model. This would involve paired right and left lungs acting as a control and intervention arm. Larger numbers than what was available for our study would be required to account for the variability between donors and often the individual lungs themselves due to single lung trauma or pathology.

Any study focussed on the nitric oxide system needs to consider the delicate balance between the beneficial and potentially detrimental effects of this vascular homeostatic regulator. NO is generated by NO synthases (NOS) that catalyse the conversion of l-arginine and O2 into l-citrulline in a complex reaction. There are three isoforms of NOS: the neuronal NOS (nNOS), the inducible NOS (iNOS) and the endothelial NOS (eNOS)[283]. Endothelial NOS (eNOS), is thought to be cell protective and can be found in vascular endothelium where is maintains vascular dilation and permeability. Inducible NOS is expressed by inflammatory cells such as neutrophils and macrophages and is induced by bacterial lipopolysaccharide, cytokines, and other agents. iNOS is constantly active once it is expressed and its regulation is independent of intracellular Ca2+ concentrations. The eNOS/iNOS balance may have important implications in vascular, pulmonary homeostasis and EVLP could act as a platform to study this interaction.

In any future studies of sildenafil and its role in lung ischaemia reperfusion injury, it would be useful to investigate the baseline levels of iNOS and the changes observed during perfusion and following an intervention. RNA studies of iNOS and eNOS mRNA between pre and post EVLP samples would be interesting, as this has already been studied in the rat lung showing that 12 hours of cold preservation had no effect on iNOS mRNA expression, however, iNOS was increased significantly 2 hours after reperfusion [284, 285]. Increased iNOS expression has also been studied in the context of acute lung rejection following transplantation with higher NO production being described during the early phases of this severe complication[286, 287]. Worral *et al.* demonstrated in the rat model that inhibition of iNOS prevented impaired gas exchange function of the transplanted lung and attenuated the histological changes of acute rejection[288].

It is known that overproduction of NO via iNOS and oxidative stress may lead to reactive oxygen and nitrogen species formation and vascular dysfunction during acute lung injury[289, 290]. Measuring the accumulation of the reactive nitrogen species, peroxynitrite via the marker nitrotyrosine could help determine the extent to which NO production could be contributing to alveolar capillary damage and increased pulmonary vascular permeability. Another area of interest is the concept of eNOS uncoupling which has been postulated to contribute to oxidative stress generation during lung IRI via a shift towards ROS production. A study by Gielis *et al*. reported that that eNOS uncoupling contributes significantly to pulmonary oxidative stress[291].

With EVLP it is possible to study the effects of time of administration of a potential therapeutic agent. A drug could be administered in the preservation solution with the organ flush as a pre-conditioning agent, given during perfusion at various time-points or given to the recipient following transplantation. This timing seems to be of importance when investigating the NO pathway and further studies could provide more clarity on this area of discussion.

We did not experience the same findings that have been reported in a number of animal studies showing beneficial effects of sildenafil in donor lungs [198, 204, 206]. This highlights the importance of studies using human tissue in a “real-life” setting where the lungs and injury represent what is seen clinically. This study investigated lungs that had already been turned down for transplantation and therefore represent a very specific cohort of poor quality or very extended criteria donor lungs.

This work emphasises that EVLP is a therapeutic platform by which a range of cell or drug therapies could be applied. There have been several studies focussing on EVLP as a therapeutic platform, and our study will add to this collection[82, 102-104, 111, 292, 293]. EVLP offers a drug delivery platform that avoids systemic toxicity and administration via the inhaled or intravenous route. There is huge potential for this platform to be used in the future for the reconditioning and recovery of donor lungs where their function is sub-optimal.

EVLP is now considered a valid clinical and research tool, with a vast, yet untapped potential to widen the donor pool. EVLP research has taken a new exciting direction towards investigating potential therapeutic agents that have immune modulating and reconditioning effects. One method of improving the utilisation of research donor lungs,

with regards to therapeutic testing, is to develop a Lobar-EVLP model. Our Newcastle group have been developing a Lobar EVLP which takes a lung pair, divides them into a right and left single lung and subsequent lobes, all of which are perfused as a separate system. There are numerous potential advantages to using such a lobar model over the whole EVLP system that we currently familiar. When using a lobar model as a therapeutic drug platform, multiple dosages of drugs, with only a fraction of the total dosage needed for the investigation can be used simultaneously. This both increases efficiency and reduces associated costs. This model also addresses inter-individual variances which can affect datasets. Our experience in EVLP of human donor lungs deemed unsuitable for transplantation has been that there is considerable variation between each lung pair. Variation can be the result of several factors: the cause of death, type of donor (DBD versus DCD), length of ischaemic time and donor demographics including age, gender, smoking status and co-morbidities. The lobar model circumvents and controls these variables by allowing both the control and intervention lobes to be from the same individual. Upon validation of the porcine lobar EVLP model, our group has moved onto the development of a human lobar perfusion model and internally paired testing of novel therapeutics. To date, our group has carried out twelve porcine lobar perfusions and three human lobar perfusions. Moving forward, the lobar EVLP model will provide a therapeutic delivery platform which will be ideally placed to assess a vast range of donor lung interventions.

In the last twenty years, EVLP technology has been transformed from an experimental method of assessing DCD lungs of unknown quality to a unique platform for the evaluation and reconditioning of donor lungs throughout clinical centres worldwide. This thesis joins the EVLP literature at a time where the EVLP technique itself has been well established and where the focus has been shifted towards utilising this platform for assessing donor lung function and treatment with a range of therapies.

My work has demonstrated the importance of maximising the analysis and knowledge gained from hugely precious human samples. With each EVLP carried out during DEVELOP-UK and sildenafil study, a donor's family had offered consent for samples to be collected and stored. It is vital that as a research team we are conducting responsible, sensitive and professional collection and handling of all human tissue. My studies have shown that further research questions can be answered well beyond the end of the original study period and that by following a

precise sampling protocol, the quality of samples does not need to be compromised. The samples from my studies will offer answers to future research questions and be utilised to aid other members of the BTRU group. I feel it is imperative that as much information is gained from each sample to respect the donor from which they belonged.

Over the past two years, along with the BTRU lung perfusion group established a reliable and efficient EVLP model for research purposes. We have adapted equipment, optimised protocols and modified consumables to ensure a cost-efficient model moving forward. This EVLP model will act as the constant when moving into future therapeutic studies and we now have confidence in its ability and limitations. The sildenafil study has been important in highlighting potential pitfalls in a therapeutic study. It emphasised the importance of the experimental design and the variability that can be encountered in a human study. It also highlighted that as compared to an animal study, the number of experimental repeats is controlled externally and therefore project timeframes often need adjusting with a degree of flexibility.

As with all areas of medicine, the future of diagnostics and therapeutics lies in personalised medicine. We are naïve to continue to think that “one size fits all” and our aim for the next 20 years of EVLP research should be to explore the lengths that we can go to in individualising each transplantation. From matching the donor to the recipient at a genetic level to assessing the quality of each donor lung pair at a cellular and molecular level to treating and reconditioning each lung depending on what therapies are required. We should be inspired by the pioneers of this field and change the way in which we think about transplantation. We should aspire to days where immunosuppression with toxic drug regimens are no longer required because the donor immunogenicity has been wiped out completely. We should aim for an era where no individual with end-stage lung disease is denied the opportunity to be transplanted.

My work adds to the bank of studies carried out locally and has established a research model in Newcastle which will allow for EVLP studies to continue. It is important for Newcastle to remain a driver in this area and maintain the respected position it holds in this field.

One aim of our team is to create an organ perfusion hub for clinical and research purposes. A hub which would allow for perfusion spanning lungs, hearts, pancreas, kidneys and livers. Through centralising perfusion in a designated perfusion hub, a level of expertise would be advanced. In the future, organs deemed unsuitable for transplantation could be sent to the Perfusion Hub for evaluation and reconditioning. They could then be sent out to other centres for transplantation. A centre for excellence would be created by centralising all perfusion activity and developing the skills and knowledge of the local team.

Regardless of the format by which EVLP research and clinical activity takes over the next ten years, it is essential that it continues. The overarching aim of the research I have carried out is to contribute to the work being done to increase the number of lungs available for transplantation. Although not a clinical study, I feel that by continuing EVLP research in Newcastle and by answering research questions an excitement and interest in this area has been renewed.

With all research projects involving human tissue, there are hurdles and barriers to be encountered. I was naïve to these coming from a clinical background and lacking any previous experience in obtaining donor tissue for research studies. The time needed to navigate a project from its very beginnings to first recruitment should never be underestimated. Despite the bulk of ethical, R&D and RINTAG approval being secured by the time of my joining the team, we encountered unexpected delays in final approvals. We encountered a 9-month delay in commencing any prospective human studies which was understandably frustrating. These barriers are often avoided in other countries, for example in Scotland one HTA licence is required for all hospitals whereas in England each hospital must have its own licence for the removal of human tissue.

With any project, there are limitations to carrying out the volume of work that was first proposed. In our initial proposal, we anticipated 40 human donor lungs to be obtained through the specific BRTU consenting by the Northern SNOD team. Within the period of study, specific consent led to donor lungs being removed for research purposes on one occasion. Generic consent, whereby the lungs had already been removed and were turned down for transplantation following removal, accounted for the majority of our research activity. It is clear that obtaining specific study consent can be achieved; however, the study was then reliant of the mobilisation of the cardiothoracic team (attending for the heart) and the donor proceeding for retrieval (often not possible in

DCD donors). These factors are out with the control of the research team and despite huge support from the Northern Specialist Nurses for Organ Donation (SNOD) team, National Organ Retrieval Service (NORS) NORS team, Research, Innovation and Novel Technologies Advisory Group (RINTAG), and NHS-BT these factors could not be overcome during this period. The research team acknowledged the difficulties facing recruitment early on within the project and steps were made in conjunction with both NHS-BT and RINTAG to expand into new sites both within the Northern region, but also the North West. It is estimated that by adding new sites within this region, an extra 70 donor lungs per annum would be available and eligible to be entered into the study.

The most important consequence of obtaining lungs via generic consent only was the inevitable longer lengths of cold ischaemia. With donors via study specific consent, the lungs would be transported from an area up to 60 miles away from the perfusion lab, compared to 270 miles when donor lungs via generic consent. Length of cold ischaemic time undoubtedly affects the function of donor lungs, and this will need to be considered for future studies.

During my research period, the EVLP perfusion lab was set-up at the Transplant Regenerative Medicine Facility at NHS-BT Donor Centre, Holland Drive Newcastle. All previous EVLP work, including EVLPs, carried out in Newcastle as part of DEVELOP-UK had been performed in the research laboratory in the Institute of Transplantation, Freeman Hospital. Moving all EVLP research out with a clinical location and environment had both benefits and barriers. The perfusion lab at NHS-BT was essentially a blank canvas for which we could create the fit for purpose EVLP lab that was required. Following all necessary risk assessments and purchasing of consumables, it quickly became a space designed solely for the teams' work. By moving from the clinical space at the Freeman, there was far less interruption from other research teams and less concern about how those other researchers may feel about the nature of our sensitive work with human organs. One downfall of being out with the hospital was losing the ability to call upon the expertise of the perfusionists, cardiothoracic surgeons and medical physics team whenever needed. We also took for granted the systems already in place in a hospital setting that allow for EVLP research to be carried out, for example, gas cylinder storage and piped oxygen and medical air. By moving to the facility at NHS-BT along with the kidney and pancreas

teams, we have been able to consolidate and streamline equipment, consumables and storage which should promote more efficient and cost effective ordering in future. EVLP research comes with an expensive price tag compared with many other projects being carried out locally. In Chapter 2 I laid out the costings breakdown, and this highlights that the cost is dictated by the expensive circuit disposables and the cost of perfusion fluid. We have developed ways to reduce costs by substituting elements of the circuit with cheaper alternatives and making “homemade” perfusate solution. There is no doubt that accurate costing will play a crucial part in planning any future EVLP research and grant application.

A major hurdle during the course of my research has been the variability of the porcine lungs obtained from Thompsons Meat Ltd. By using lungs procured from pigs culled as part of the commercial food chain we gained access to a weekly supply of lung pairs that would otherwise be discarded by the meat trade. This gave us an important training resource that came from a free of charge, regular and dependable supply stream. Porcine lungs were used due to their similarity in physiology and organ development to humans. The major downfall with porcine lungs from this source was the poor quality and huge variability of the lungs week to week. There have been many learning points from repeated procurements at the abattoir. There were often lung contusions, lacerations and damage to the pleura visible on being presented the lungs; most likely due to the method by which the organs were removed from the pig.

The lungs often showed gross, frank pulmonary oedema from the outset and it is thought that this was caused by shear stress from the removal of the lungs from the pig thoracic cage, leading to damage to the vasculature and loss of vascular integrity. Another problem with porcine abattoir procurements is the variability of the warm ischaemic times. Other groups have used the animal model for experimental EVLP work using a controlled Schedule 1 termination and utilising an anaesthetic and surgical team for procurement. This eliminates variability in procurement method and ischaemic times, however, comes at the cost of purchasing and housing animals for research. It also raises the ethical issue of culling animals for research versus using organs that would be otherwise discarded as part of the food chain.

Conclusions

The major conclusions that can be drawn from the research performed in this thesis are as follows:

1. RNA sequencing analysis is an effective tool that can be utilised to assess changes in the gene signature in human donor lungs during perfusion. Transcriptomics can be used to generate potential biomarkers that identify lungs suitable for transplantation following *ex-vivo* perfusion. There were more than 700 genes were differentially expressed pre- and post-EVLP, and many of these are involved in immunological processes. There are differences in the transcriptome between lungs that were considered to be transplantable and those that were declined in the baseline lung tissue samples obtained prior to EVLP. The TNF-α signalling via NFκB pathway is significantly upregulated in declined lungs.
2. In extended criteria donor lungs which have been turned down for immediate transplantation, there are differential inflammatory and tissue injury marker profiles between DBD and DCD lungs. IL-4, IL-6 and IL-12 were significantly higher in tissue samples from DBD lungs. IL-α and IL-1β were detected in significantly higher levels in the perfusate of DCD lungs at as early as 15 minutes and throughout reperfusion. This study also showed significant differences in the expression of angiogenic factors between DBD and DCD lungs with higher levels of Placental growth factor (PlGF) and soluble Fms-like tyrosine kinase receptor 1 (sFlt-1) expression in the perfusate of DBD compared with DCD lungs.
3. EVLP is an effective platform for novel therapeutics aimed at the reconditioning of human donor lungs deemed unsuitable for transplantation. The vasodilatory agent sildenafil failed to improve lung physiology or attenuate pro-inflammatory cytokine expression when given during EVLP.

Implications and Future Directions

The studies contained within this thesis do not stand in insolation and are part of the exciting work ongoing within the NIHR BTRU ODT. Below follows a brief summary of the major ongoing investigations outlining the future direction of our research:

Using transcriptomics to generate biomarkers that identify kidneys suitable for transplantation following ex-vivo perfusion

Chapter 3 of this thesis demonstrated the feasibility of using transcriptomics as a method for understanding the pro-inflammatory signal from the perfused donor lung and investigating potential predictive biomarkers of EVLP performance and post-transplantation outcomes. Similar studies are being carried out by Dr Menna Clatworthy and Dr John Ferdinand using samples from EVNP of the kidneys. These studies will highlight the major differences and similarities between the genetic alterations taking place during lung and kidney perfusion.

Modelling leukocyte-endothelial interactions in ex-vivo organ perfusion

In a BTRU sponsored PhD project from our group, PhD student Mr Tom Pither, aims to further our understanding of the relationship between leukocyte activity and organ endothelial status post-perfusion.

The project at this point has two major aims:

1. To investigate the microvascular endothelial cells from lung and kidney and their response to activation by IL-1β. His work will focus on the microfluidic flow assay, *Cellix* to quantify neutrophil adhesion, rolling, and transmigration to the endothelial monolayer when activated with interleukin-1β, as well as assessing subsequent extent of neutrophil activation.
2. *Ex-vivo* modelling of endothelial/neutrophil interactions, observing the migration of labelled neutrophils through sections of lung and kidney tissue from animals and human donors in response to IL-1β stimulation and its targeted inhibition.

EVLP is currently being utilised as a platform for studying and investigating leukocyte behaviour, activity and final destination when added to perfusate. Tom has been successful in demonstrating the adhesion of labelled neutrophils using immunofluorescence microscopy and graphically representing the number of labelled cells in perfusate across a time course of perfusion. Recent results show that the initial infusion of neutrophils into the circulating perfusate results in a spike in cells counted,

and as expected, the majority of binding can be seen to occur within the first 5 minutes of neutrophil infusion. This is then followed by a dramatic fall in the number of circulating cells over the following 5 minutes of perfusion. This decrease continues until around 20 minutes of perfusion, whereby a plateau is reached with numbers remaining relatively static until perfusion is ceased after 120 minutes. This follows the expected dynamics of neutrophil adhesion and provides a clear and obvious mechanism which can potentially be modulated by the addition of therapeutics.

Future studies of leukocyte-endothelial interactions in *ex-vivo* organ perfusion will utilise the lobar EVLP model and focus on testing potential therapeutics. Previous studies by our group, have demonstrated that perfusate IL-1β levels were strongly associated with early graft performance and could in the DEVELOP-UK multicentre study, with high sensitivity and specificity, diagnose both EVLP assessment failure and early post-transplant mortality[83]. Andreasson *et al*. demonstrated that by inhibiting the action of IL-1β, using an IL-1RA or more potently an IL-1β neutralising antibody, both the upregulation of key endothelial surface adhesion molecules and neutrophil adhesion to pulmonary endothelial cells could be significantly reduced *in-vitro.* Future work will move to using a lobar EVLP model to investigate the effects of therapeutic inhibition of IL-1 during EVLP, using the anti-IL-1 receptor antagonist Anakinra (Kineret, Swedish Orphan Biovitrum, Stockholm, Sweden).

The use of cell-based therapy in ex vivo reconditioning of lungs prior to transplantation

EVLP allows for perfusion over a prolonged period of time, and this offers a unique platform for administering pharmaceuticals, gene and cellular therapies that can repair lungs and target the prevention of PGD before transplantation occurs. Human Amniotic Epithelial Cells (hAECs) have recently attracted attention in the field of regenerative medicine as potential cell therapy, due to their multipotent ability, anti-inflammatory functions and low immunogenicity[294]. These cells can be isolated from the human term placenta following elective caesarean(C)-sections, which would usually have been discarded. Human amniotic epithelial cells or their conditioned media have the potential to recondition human lungs during ELVP by reducing pulmonary vascular endothelial activation and limiting the leukocyte-endothelial interactions after organ reperfusion.

In a BTRU sponsored PhD project from our group, PhD student Chelsea Griffiths aims to:

1. Establish a robust hAEC isolation technique, fully characterise the isolated cells and collect conditioned media.
2. Investigate the anti-inflammatory and immunomodulatory properties that hAECs have on leukocyte-endothelial interactions.
3. Evaluate the biological and physiological effects of hAECs or their conditioned media have on human lungs during EVLP.

A Near Point of Care Cytokine Test - Evalution™

My predecessor Mr Anders Andreasson had established a collaboration with a Belgian company to evaluate the feasibility of using their Evalution™ biomarker assay platform (MyCartis, Zwijnaarde, Belgium)[295]. One of the obstacles in the standard EVLP assessment is the time before a decision on transplant suitability can be made, currently around four hours. For any biomarker technology to have real impact and clinical relevance it has to be highly accurate and able to deliver a result available to the clinical team at the time of decision-making. The previous work on predictive biomarkers has been based on MSD, and ELISA techniques which although highly sensitive are limited by them both utilising passive binding between antigen and antibodies, and therefore requiring incubation times of at least 3-5 hours. This is adequate for retrospective analyses, but not applicable for clinical use.

MyCartis has carried out preliminary feasibility studies focussing on interleukin-1β assays. The assay uses the “co-flow” principle of simultaneous incubation of sample, detection antibody and reporter molecule to reduce the manual interventions to a few pipetting steps, and reduces the assay time to approximately 30 minutes. Further on-going collaboration involves analysis of perfusate samples from our sildenafil cohort with corresponding MSD data available. Further validation of their assay is required, and they plan to send a ready-to-use assays and assay kits to Newcastle.

Mitochondrial biology of lungs undergoing ex vivo lung perfusion

There will be future collaboration with the mitochondrial biology cross-cutting BTRU theme, led by Dr Mike Murphy and PhD student Anja Gruszczyk. Utilising EVLP perfusate and lung tissue samples future studies will investigate the role of mitochondrial oxidative damage in ischaemia reperfusion injury that inevitably accompanies organ transplantation[296]. Following on from work on EVNP of

kidneys[297], future studies will focus on the protection against mitochondrial oxidative damage in the lungs by the mitochondria-targeted anti-oxidant Mitoquinone or MitoQ™. Future collaborative work will involve investigating the role of succinate accumulation in lungs during storage and subsequent damage on reperfusion.

Summary

Lung transplantation can be a life changing procedure for selected patients with end-stage lung disease. It is, however, currently limited by low donor and poor donor lung utilisation rates. EVLP demonstrates promise as a method to better evaluate lungs during the ex vivo phase of transplantation. Moreover, drug delivery to lungs during EVLP offers new therapeutic potential with the ambition of reconditioning lungs initially turned down for transplantation. There is huge scope for EVLP's potential for reconditioning lungs in the future as EVLP offers a unique platform tailored to this clinical and investigative work, aiming to find novel ways to increase the donor pool and to make lung transplantation a reality for more waiting list patients with life threatening lung disease. The next chapter in the history of EVLP is an exciting and ambitious one with future goals focussed on the development of specialised national organ perfusion centres to facilitate ex vivo perfusion of organs; the reconditioning of ex vivo organs with cell and regenerative therapies and personalised medicine whereby donor lungs are somehow programmed for each individual recipient. It would the naïve and foolish to consider EVLP as the only method of increasing the donor lung utilisation rate as this will continue to be dependent on a number of important factors. There will continue to be an ongoing drive to increase the number of people registered on organ donor registries worldwide, with the impact of the "opt out" schemes eagerly anticipated. Donor management within intensive care units is an important area of ongoing research along with the utilisation of DCD donor lungs. The next 20 years poses huge potential to better our understanding of the fundamental pathophysiology of ischaemia reperfusion injury and investigating therapeutic targets. This ongoing research is vital to those patients waiting on the lung transplant waiting list becoming more unwell as time passes and to patients following lung transplantation who suffer primary graft dysfunction with its associated morbidity and mortality.

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APPENDIX A

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Morrison MI, Pither TL, Fisher AJ. Pathophysiology and classification of primary graft dysfunction after lung transplantation. *Journal of Thoracic Disease*. 2017;9(10):4084-4097. doi:10.21037/jtd.2017.09.09.

APPENDIX B

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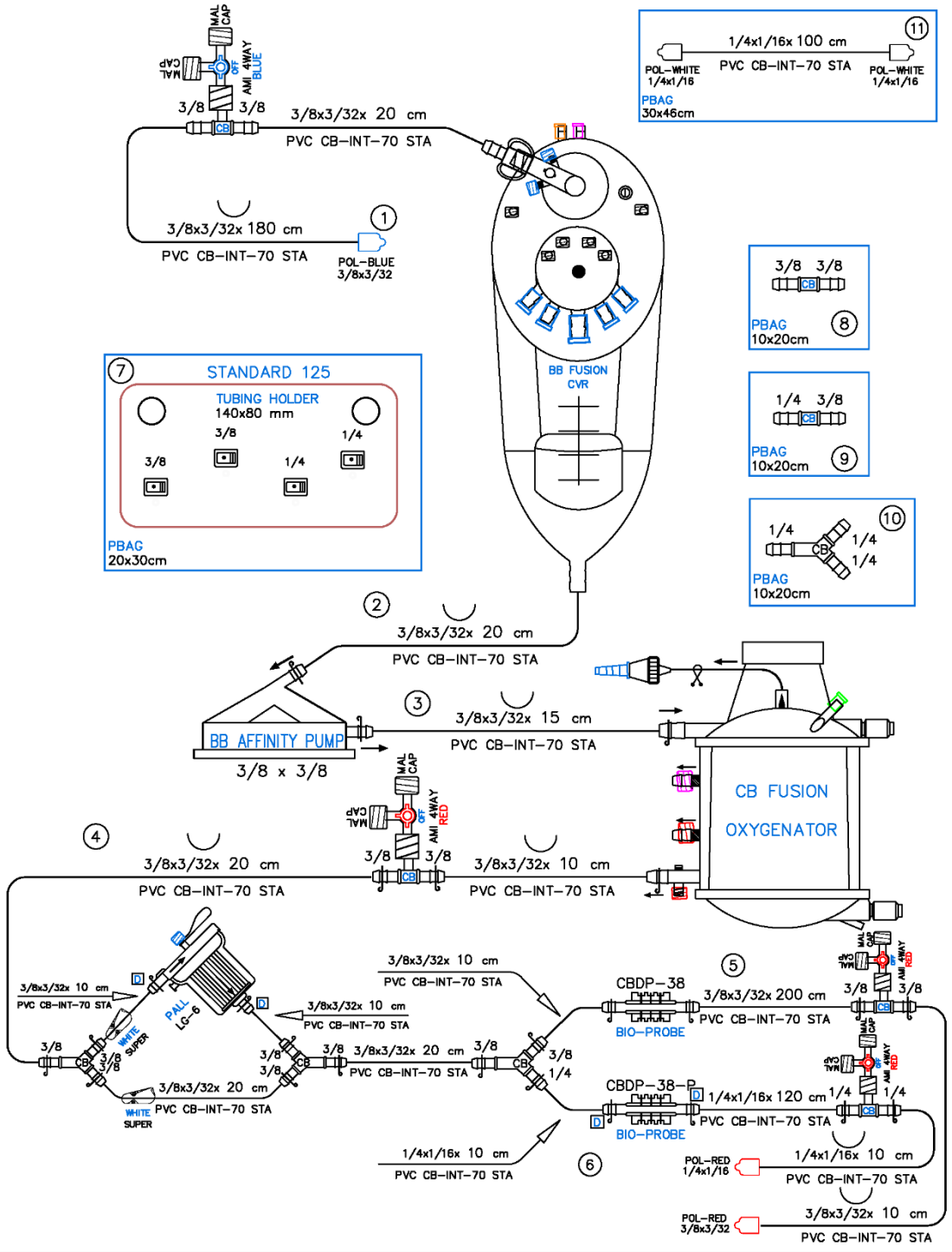
Medtronic EVLP circuit design

Medtronic

Custompack Specifications



Pack Title : CB PERFUSION SUPPORT SET 1/B

Att.refcode : 161100110101100

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DRAWING NUMBER

M444429B

APPENDIX C

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EVLP costings

Total cost per EVLP £4668

Consumable	Supplier	Catalogue Number	Amount per isolation (g/ml/item)	Cost per EVLP (£)
Perfusion				
Lung perfusion Set up kit	Medtronic	444426C	1	680
Steen solution	XVIVO PERFUSION	19004	2500	3504
Cannulae	XVIVO PERFUSION	19022	3	266
Scalpel no10	Brosch Direct	NS1010	2	0.55
Suture 4.0 prolene	Medisave	SKU:W8020T	2	6.324167
Prolene 5.0 Suture	Medisave	SKU:W8010T	2	7.3575
Ventilation				
Ventilation Tubing	Intersurgical	1528000	2	3.9
Ventilation Y piece	Intersurgical	190000	1	1.813333
Ventilator Catheter Mount	Intersurgical	3508000	1	0.7
Ventilator Filters	Teleflex	19402T	1	0.42
Suction tubing	Medisave	ASP010/SET	1	0.446
Medical Air size J	BOC	191-J	0.25	4.64
Oxygen Size W	BOC	1-W	0.5	2.38
Special Mixed Gas CO2/N2 (7% CO2)	BOC	152295-L-C	0.5	72.585
Sample collection				
ISTAT cartridges CG4+	Abbot Laboratories	03P8525	15	63
50ml Luer Lock Syringe	Brosch Direct	NS3666	1	0.462
Woven Swabs	Brosch Direct	DW6412	4	0.022667
Sterile Containers 30ml	SLS	CON7502	4	1.09
Syringe 10ml Luer lock	Medisave	SKU:SYR912	15	2.2485
Needle 21gage Green Terumo	Medisave	SKU:8N-2138R	15	0.3525
Cryobox	Starlab	E2383-5004	1	7.573333
20ml syringe BD plastipack	Medisave	SKU:SYR213	7	0.874417

APPENDIX D

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Study logistics flowchart

Appendix 3: EVLP Study – Retrieval and Logistics Summary

In order for Lungs to be procured explicitly for research use in this EVLP study the following must be met:

1. SNODs have obtained generic and study specific research consents.
2. Lungs are not accepted by any centres for clinical use.
3. Donation occurs in a designated study site with an HTA licensed facility
4. A donor CT retrieval team has already been mobilised for a potential transplant, is present and other functions of the team, particularly heart retrieval, are not impeded.
5. Licenced transport will be arranged by the Research team to transport research lungs and any retrieval staff who have stayed on back to Freeman Hospital. The Lungs will be couriered back to the locked fridge in ward 38, Freeman Hospital.

Logistics Flow Chart

The SNOD will inform the on-call cardiothoracic transplant co-ordinator at Freeman Hospital that donor lungs have been authorised for research if not fit for transplant.

The transplant co-ordinator will communicate this to the CT retrieval team attending that donor; the retrieval team should always travel and carry with the lung retrieval kit/equipment to every retrieval.

The on-call cardiothoracic transplant co-ordinator calls the Research team to highlight the potential donor;

Morvern Morrison (1st contact) 07919 380 271 and/or **Bill Scott 07530 743 693**

Lungs may be retrieved for research purposes if they have already been turned down in-situ (e.g. due to history, arterial blood gases or chest x-rays) or if they are removed and turned down on visual inspection at explantation.
As soon as the lung retrieval has started (or aorta cross clamped), the SNODs will inform the research team of when and where the courier must be waiting. The SNOD is responsible for handing the lungs to the courier.

There are a few specific specific scenarios this listed below:

When Abdominal Organs are accepted for clinical use;

If the time to be taken before cross-clamp is less than one hour, and if there is no need for the team to return to base because of a back-to-back retrieval, the team will wait for cross-clamp and retrieve the lungs.

When the Heart is accepted for clinical use;
Rapid dispatch of the heart is the priority, but if a taxi can be arranged, and the team are not needed for the cardiac implant or a back to back retrieval, they will stay and retrieve the lungs.

DCD donor;
Team will not wait beyond 2 hours. If NRP is used in DCD retrieval, the cardiothoracic team will not retrieve the lungs until this process is finished. Clinical organs will not be jeopardised for this research study. The decision to wait until the abdominal team are finished is at the cardiothoracic team's discretion.

APPENDIX E

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DEVELOP-UK EVLP standard operating procedure

DEVELOP-UK EVLP Set- SOP

Location

EVLP was performed in an operating theatre, a suitably designated clinical area that fulfils the necessary infection control precautions.

Equipment

- **Vivoline LS1 evaluation unit**: An integrated roller pump (Jostra), heater cooler unit, gas system, monitor and control unit with associated software package.
- **Vivoline LS1 disposable lung set**: Includes an oxygenator (Capiox), LDF leukocyte filter, pressure sensors and temperature probes.
- **93% Nitrogen and 7% CO2 gas mixture cylinder on a trolley**: A full cylinder contains 10000 litres and each EVLP run requires at least 2000 litres of gas mixture (3 hours).
- **Oxygen supply**: From the wall outlet.
- **ICU standard ventilator:** Model that measures minute and tidal volumes, lung compliance / resistance and airway pressure.
- **Arterial blood gas machine**.
- **Standard thoracic surgical instrument tray**.
- **Sterilised bronchoscope**: With suction tubing and lavage traps, 5, 10 and 20ml syringes.
- **Sample collection disposables:** Includes Duet-stapler devices for lung biopsies, specimen pots for tissue, test tubes for perfusate and ice bucket or refrigerator for sample storage.

Medications

- **Saline 0.9%:** 240 ml for 2xBAL and additional 100 ml for flushing pressure transducers.
- **Steen Solution:** At least 4 x 500 ml bottles.
- **Packed Red Blood Cells:** Request 3 packs of red blood cells from the blood bank, either universal donor (O negative) if >1 potential recipient identified or cross matched to the single known recipient.
- **THAM/TRIS solution:** Use a preparation containing Trometamol 3.0 mmol/ml in a quantity as directed by base deficit to a Base Excess +/- 3 and pH of 7.35 – 7.45

- THAM is available in various dilutions (ranging from 3.6% (Abbott, Köhler) to 36.5% (Braun) or 40% (Fresenius-Kabi or Addex-THAM) so caution is advised in measuring the correct amount. A 3.6% solution of THAM contains a concentration of Trometamol (active ingredient) of 0.33mmol/ml. A 40% solution of THAM contains a concentration of Trometamol (active ingredient) of 3.0mmol/ml. When buffering Steen solution in the circuit use 3mmol of THAM per minus unit in base deficit. This will be 1ml per minus unit base deficit for the 40% THAM preparations and 10ml per minus unit base deficit if the 3.6% THAM preparation is used. A THAM preparation of between 3.0 – 3.3 mmol/ml is strongly recommended and use of lower concentrations strongly discouraged due to the dilutional effects on Steen Solution of adding large volumes of a lower concentration THAM.

- **Heparin:** 10,000IU unfractionated.
- **Methylprednisolone:** 500mg.
- **Antibiotics:** Meropenem 500mg is the default antibiotic if there is no documented recipient allergy to ß-lactams. If donor airway cultures known, other antibiotics can be used after discussion with microbiology.
- **Amphotericin B:** 10 mg for injection (Amphocin, Fungizone or equivalent).
- **Actrapid Insulin and 10% Glucose Solution:** Possible requirement for 10ml of 10% Glucose solution and 20 IU Actrapid Insulin but not routinely used.

Priming the Circuit

- Connect the disposable kit to the evaluation unit according to user manual instructions.
- Flush the pressure transducers.
- Connect the water to the heater-cooler unit, open the water bag.
- Start the ***priming phase*** on the screen. Check the oxygenator for leakage.
- Calibrate the pressure transducers.
- Fill the system with 2.0L Steen solution (4 x 500ml bottles).
- Calibrate the pump occlusion by following the screen instructions.
- Recirculate perfusate at 15°C set flow to 2.0 L/minute and maximum perfusion pressure at 10 mmHg.
- Connect the gases.
- Add 10,000IU of unfractionated Heparin to circulating Steen solution.
- Add 1-2 units of Packed Red Blood Cells.

- Prepare 500mg dose of Methylprednisolone.
- Make up Antibiotics and Amphotericin B according to manufacturers' instructions.
- Add Methylprednisolone, Antibiotics and Amphotericin B to the circulating perfusate.
- Do a perfusate blood gas analysis and correct the base deficit with THAM to a Base Excess +/- 3 and pH of 7.35 – 7.45. Add 3 mmol THAM per minus unit in base deficit. **The blood gas needs to be temperature corrected for 15ºC**.
- Note haematocrit of perfusate on blood gas. Target is 10-15% so add more red cells if needed.
- Check perfusate glucose and potassium concentration via blood gas analysis; if glucose <5 or >20 mmol or potassium >7mmol correct with Insulin.
- Repeat the perfusate gas prior to connecting the lung for any further corrections.
- The perfusate, pharmaceuticals and gases should be circulated for ≥15 minutes before connecting the lung.
- If satisfactory, start the ***reconditioning phase***.

Reconditioning Phase

- Surgical dissection is performed to allow placement of the donor lung onto the Vivoline EVLP circuit in the covered organ bath to maintain humidity.
- Before connecting the lung to the circuit, take ***Lung Biopsy #1*** from the RML or Lingula and collect ***Perfusate Sample P0*** (See separate Sample collection SOP).
- Cannulate the main pulmonary artery with the quick-fix pre-fashioned cannula and open the shunt to the inflow cannula.
- The left atrium is left open and visualised to ensure a smooth flow of perfusate.
- The LA temperature probe and sampling line is secured in place equal distance from the 4 pulmonary veins.
- Where possible the trachea remains clamped with lungs partially inflated with 50% FiO2 while the quick-fix ventilation tube is secured in place. This prevents collapse of the lungs and development of atelectasis prior to the ventilation.
- Set the pulmonary artery (PA) pressure to a maximum of 15 mmHg.

- Perform de-airing of the circuit with the shunt open at a flow of 0.5L / minute for approximately 2 minutes until fully de-aired. Leave the shunt open.
- Set temperature to 32^{o}C. The system will warm up the lung automatically with a maximum delta temperature of less than 8°C.
- If initial perfusion is uneventful, increase the PA pressure limit to 20 mmHg and flow to maximum.

  The recommended max flow is 70 ml/kg IBW /minute. With a cold lung the pressure will limit the flow, when the resistance in the lung goes down the flow will increase over time.

- When temperature has reached 25^{o}C close the shunt.
- At 32^{o}C remove the clamp on the trachea and before commencing ventilation, perform a bronchoscopy and collect ***BAL sample #1*** from either RLL or LLL using 120 ml 0.9% NaCl with the bronchoscope in a wedged position (See separate Sample collection SOP).
- Commence mechanical volume-controlled ventilation at 32^{o}C with a protective ventilation strategy:
    - Set inspired oxygen (FiO_2) to 0.5 (50%)
    - Set respiratory rate (RR) initially to 5 or 8 breaths/min according to donor IBW
    - The ideal body weight calculation formulae used to determine the tidal volumes in protective lung ventilation are:

  IBW (kg) for men = [(height (cm) -154) x 0.9] + 50

  IBW (kg) for women = [(height (cm) -154) x 0.9] + 45.5

    - Set Minute volume (mV) initially to 1 L/min
    - Set PEEP at 5 cmH_2O
    - Set I:E ratio 1:2 and inspiratory pause at 10%
    - Keep peak airway pressure <20 cmH_2O
- Increase temperature from 32^{o}C to 37^{o}C.
- Increase the mV in 1 L/min increments gradually as lung warms to 37^{o}C; **mV should not exceed 1.5X the flow**. Continue to keep peak airway pressure (Paw) < 20 cmH_2O. Increase the RR, as mV increases, up to a maximum of 15 breaths/min to keep tidal volume <7ml/kg IBW.

- At 32°C the perfusate flow is usually lower than the set value, please note the flow as the lungs warm to 37°C as the **minute volume should not exceed 1.5X the flow**.
- If uneventful and once 37°C reached, mV can be increased **gradually** up to a maximum of 100 ml/kg IBW/ min but the tidal volume should not exceed 7 ml/kg IBW.
- If persistent atelectasis present, perform a recruitment manoeuvre by transiently increasing PEEP from 5 cm H_2O in increments of 1 cm H_2O for a few breaths to a max of 12 cm H_2O while always keeping Paw <25 cm H_2O. Note flow will fall significantly during increase in PEEP. After recruitment return PEEP to 5 cm H_2O.
- Flow, PVR, Lung Compliance and PA pressure to be documented once lungs reach 37°C and flow stabilised.
- After lungs have reached 37°C and flow and ventilation stabilised, shift to the ***evaluation phase***.

Evaluation Phase

- Once re-warming is complete and target perfusion established, the function of the donor lungs undergoing EVLP can be assessed as specified in the study protocol.
- Disconnect the oxygen from the perfusion system prior to evaluation.
- Once the perfusate is deoxygenated and confirmed on blood gas analysis, perform recruitment manoeuvres as above and set the ventilator for evaluation as below:
    - Increase FiO_2 via the ventilator from 50% to 100%.
    - PEEP can be increased to 8 cm H_2O for a short period.
    - Maximum mV should not exceed 100 ml/kg/min (donor IBW)
    - Keep peak airway pressure Paw <25 cm H_2O
- RR can be adjusted between 12 and 15 breaths/min to maintain V_T up to a maximum of 7 ml/kg (donor IBW)
- Perform blood gas analysis 15 minutes after FiO_2 is increased to 100% to assess venous and arterial pO_2 values. Blood gas analyses should be performed from each pulmonary vein as well as a mixed LA sample.
- Flow, PVR, Lung Compliance and PA pressure should be carefully documented on the data sheets.

- Perform a lung deflation test by disconnecting the tracheal tube at the end of inspiration. **Remember to first reduce perfusate flow to maximum of 1.5L/min to avoid alveolar oedema.** Recoil of the lungs is evaluated subjectively; global collapse of the lungs is defined as normal.
- If transplant suitability criteria have been achieved, move immediately to ***cooling phase*** for organ preservation.
- If transplant suitability criteria have not been achieved, return to the reconditioning phase.
- Perform hourly clinical assessments as documented in the protocol until a decision on suitability of the lungs for transplantation or 240 minutes of EVLP perfusion has been reached (from the time of reaching 37°C).
- During perfusion if pH <7.35 administer additional THAM to the Steen Solution™.
- During perfusion do not automatically replace Steen Solution™.

Cooling Phase

- Reduce mV by a 30% reduction in tidal volume and lowering respiratory rate to 8 breaths/min
- Set temperature to 32°C
- Reconnect the oxygen to the perfusate.
- Before discontinuing ventilation, perform a bronchoscopy and collect ***BAL sample #2*** from the same lobe, but from a different segment than BAL sample #1, using 120 ml 0.9% NaCl with the bronchoscope in a wedged position (See separate Sample collection SOP).
- Stop ventilation at 32°C, clamp trachea with lungs partially inflated with 50% FiO2.
- Set temperature to 12°C and continue to cool lungs until perfusate temperature 12°C.
- Collect ***Perfusate Sample PX***.
- Disconnect PA cannula and plug PA with special bung.
- Once perfusion has stopped and the lung is disconnected, take ***Lung Biopsy #2*** from the same lobe as Lung Biopsy #1 (See separate Sample collection SOP).
- Commence topical cooling.

- Place mat under lungs and wrap towels over lungs so that they touch the mat all around lungs.
- Connect the Y shaped hose from the cooling assembly to the lung perfusion quick connection and cover the highest point of each lung.
- Connect the remaining hose from the cooling assembly to the shunt quick connection and place over the trachea and PA.
- In the preservation phase set pump to 2.5 L/ min. Check fluid level and add more Steen Solution if necessary.
- Maintain the lungs in topical Steen solution at 6 - 8 °C on the circuit (preservation phase) until ready for transplant.

Adaption for Single Lung Reconditioning

- For cannulation, if feasible staple the contralateral PA (right PA if it is a left lung transplant or vice versa) at least 2 cm above its first branch to facilitate as much length as possible. If the pulmonary artery is too short and/or the surgeon is unable to fit the quick-fix pre-fashioned cannula, use a part of the donor's aorta to augment the cuff.
- For connection of the bronchus, please note there are 3 sizes of connection available in the disposable kit to aid achieving an effective connection. If it is a right lung EVLP, staple the left main bronchus at the level of the carina and cannulate the trachea. If it is a left lung EVLP, the left main bronchus from the level of the carina should be long enough to facilitate attachment. Ensure a seal that prevents either ineffective ventilation of fluid entering the airway.
- Set to 50% target flow i.e. 35 ml/kg body weight/minute.
- When starting ventilation, start at a minute volume of 0.5 L/min and increase to a maximum of 1.5 times the perfusate flow.

APPENDIX F

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Protocol for alternative STEEN Solution

Protocol for alternative STEEEN Solution

List of equipment/consumables needed:

- Fisher Glass Bottles – 2 litre capacity
- Scales
- Funnel
- Filter paper
- Sterile filters
- Labels
- Dextran 40 BioChemica from VWR international cat ref #A2249.0500
- Bovine Serum Albumin Insight Biotechnology catalogue numbers: sc-2323A
- Sodium Bicarbonate Sol. 7.5% Sigma Aldrich S8761
- Calcium Chloride Dihydrate Sigma Aldrich 000020 C7902
- Krebs-Henseleit Buffer with 2000 mg/L Glucose, Without Calcium Chloride and Sodium Bicarbonate Product Number Sigma Aldrich K3753

To make 2 litres of stock:

1. Fill Glass Jar (2 litre capacity) to 1800 ml with distilled water, and then mix in 2 bottles of powdered Krebs Buffer.
2. Mix gently on magnetic stirrer plate until liquid clear and no visible fragments
3. Add 56 ml of 7.5% Sodium Bicarbonate Solution
4. Add 10 g of Dextran 40 (0.5% concentration)
5. Add 140 g of BSA (7% concentration)
6. Top up to 2 litres with distilled water
7. Mix on magnetic plate
8. Add Calcium Chloride
9. Filter through filter paper into sterile jar
10. Label with “Homemade Steen”, date and initials

Constituents of Alternative Perfusate Solution

	Mol weight (g/mol)	Stock conc (mmol/L)	Weight (g) into stock	Vol stock (L)	Conc req (mmol/L)	Vol stock (ml) for 1L Steen	Dry weight to add (g)
NaCl	58.44	1560	182.33	2	114	73	0
$NaHCO_3$	84.01	250	42.01	2	25	100	0
KCl	74.55	92	13.72	2	4.6	50	0
NaH_2PO_4	137.99	120	16.56	1	1.2	10	0
$MgCl_2$	203.3	120	24.4	1	1.2	10	0
$CaCl_2$	147.02	30	8.82	2	1.5	50	0
Glucose	180.16	NA	NA	NA	11	NA	1.9818

APPENDIX G

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ELISA protocol

Solid Phase Sandwich ELISA protocol

1. *Coating with Capture*

   1a. Dilute the Capture Antibody in PBS 1:100

   1b. Add 25µl/well of the diluted Capture Antibody. 96-well microplates (R&D systems) were coated with capture antibody/PBS (1:100)

   1c. Seal the plate and incubate overnight at Room Temperature on a rocker.

2. *Blocking*

   2a. Wash all plates using the ThermoScientific Washer primed with Buffer A – 1000 ml Distilled Water with 5 tablets of PBS and 0.5 ml Tween)

   2b. Add 150 µl/well of Reagent Diluent (1% BSA and PBS – 100 ml: 1g BSA + 100 ml PBS)

   2c. Seal the plates and incubate at room temperature on the rocker for 2 hours.

3. *Standard and Sample preparation*

3a. Defrost from -80°C freezer aliquoted Eppendorf's of the appropriate standard for marker kit

3b. Prepare standard in Reagent Diluent 1:100

3c. Take sample transfer plates from -80°C freezer and defrost on ice

3d. Wash all plates

3e. Add 25 µl/well of samples and standards in duplicate.

3f. Seal the plates and incubate at room temperature on a rocker for 2 hours.

4. *Detection*

   4a. Dilute the Detection Antibody (defrost aliquoted Eppendorf's for appropriate kit from -80°C freezer) in Reagent Diluent 1:100.

   4b. Wash all plates

   4c. Add 25µl/well of the diluted Detection Antibody.

   4d. Seal the plates and incubate at room temperature on the rocker for 2 hours.

5. *Streptavidin-HRP*

   5a. Dilute Streptavidin-HRP in Reagent Diluent 1:40

   5b. Wash all plates

   5c. Add 25µl/well of the diluted Streptavidin-HRP.

5d. Seal the plates and incubate at room temperature, in the cupboard, avoiding direct light, no rocker, for 20 minutes only.

6. *Substrate Solution*

   6a. Prepare Substrate Solution (stored at 4°C) at a ratio of 1:1 of Substrate A: Substrate B

   6b. Wash all plates

   6c. Add 25µl/well of Substrate Solution.

   6d. Incubate at room temperature, in the cupboard, avoiding direct light, no rocker, for a maximum period of 20 minutes.

   6e. Observe standard columns and samples for yellow colour change. Observe for dynamic changes and variation. Ensure standards show progressive colour change.

7. *Stop Solution*

   7a. Do not wash plates at this step

   7b. Make up Stop Solution (pre-prepared 1M H2 SO4. 26.7ml H2 S04 + 473.3 ml H20)

   7c. Add 25µl/well of Stop Solution. Gently tap the plate to ensure thorough mixing.

   7d. By adding Stop Solution there will be a second colour change to blue.

8. The absorbance was read on the plates using a microplate reader set to 450nm.

9. Mean optical densities were corrected for background signals using blank wells as controls then divided by the gradient the standard curve and multiplied by the dilution factor to give an estimate of protein volume.

APPENDIX H

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Immunohistochemistry protocol

Immunohistochemistry protocol

- Pre heat sections to 60°C for 1 hour.
- De wax in xylene for 2 x 5 minutes.
- Re hydrate through graded alcohols: 1 minute each 99% IMS, 99% IMS, 95% IMS
- Block endogenous peroxidase in 0.3% Hydrogen peroxide in Methanol for 30 minutes.
- Wash well for at least 10 minutes in gently running tap water.

Antigen retrieval:

eNOS

- Place 600 ml Retrieval buffer in a white plastic bucket with vented lid, bring to boiling point.
- Using forceps carefully add the rack of slides to the boiling buffer, replace lid and return container to the microwave oven.
- Set the power control on MAX, set timer and press start
- At the end of the time period, remove from microwave and allow to cool for 15 minutes.
- Remove the rack of slides and transfer to Tris Buffered Solution (TBS)

Neutrophil Elastase (NE)

- Antigen retrieval using trypsin (pH7.6) – use water bath at 37.5°C for 20 minutes

Antibody steps:

eNOS

- Dilute the primary antibody to final concentration 1:50
- In a bioassay incubating tray containing moist tissue, apply 100µl of diluted antibody to the appropriate section.
- Incubate for optimised time period - room temperature for 1 hour and then overnight at 4°C, in the fridge for 18hours.

Neutrophil Elastase (NE)

- Dilute the primary antibody to final concentration 1:100
- In a bioassay incubating tray containing moist tissue, apply 100µl of diluted antibody to the appropriate section.
- Incubate for optimised time period - room temperature for 90 minutes

To finish:

- Use wash bottle to gently rinse the slides in TBS. Place in a metal rack in a staining trough containing 300 ml fresh TBS. Wash gently for 5 minutes.
- Repeat wash in TBS 5 minutes
- Remove excess buffer but do not allow section to dry out and apply 100 µl of the secondary antibody and incubate for 30 minutes at room temperature.
- Gently rinse slides with TBS and place in a trough of 300 ml of TBS. Wash 5 minutes.
- Gently remove excess buffer and treat sections with DAB 5-10 minutes. Mix Dako Buffer (K4010) 1 ml/1 drop DAB concentrate well immediately before use.
- Wash sections in running tap water.
- Counterstain with Carrazzi's Haematoxylin for 1 minute. Wash gently in tap water until blue.
- Dehydrate through 95%IMS, 99% IMS, 99% IMS for 1 minute each
- Clear sections in xylene
- Mount in DPX/Perfex
- Read on microscope